Pak. J. Bot., 52(5): 1857-1865, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-5(10)

THERMAL STRESS ALLEVIATING POTENTIAL OF ENDOPHYTIC FUNGUS *RHIZOPUS ORYZAE* INOCULATED TO SUNFLOWER (*HELIANTHUS ANNUUS* L.) AND SOYBEAN (*GLYCINE MAX* L.)

ISMAIL[1], ANWAR HUSSAIN[1], ASIF MEHMOOD[2], MUHAMMAD QADIR[1], HUSNA[1], AMJAD IQBAL[3], MUHAMMAD HAMAYUN[1*] AND NAEEM KHAN[4]

[1]*Department of Botany, Faculty of Chemical and Life Sciences, Abdul Wali Khan University Mardan, Pakistan*
[2]*Institute of Biological Sciences, Sarhad University of Science and Information Technology, Peshawar, Pakistan*
[3]*Department of Agriculture, Abdul Wali Khan University Mardan, Pakistan*
[4]*Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan*
[*]*Corresponding author's email: hamayun@awkum.edu.pk*

Abstract

Agricultural crops including sunflower and soybean are facing thermal stress because of rapid change in climate caused by global warming. Some innovative steps should be taken on an emergency basis to prepare plants for such unfriendly stressful conditions. Use of endophytic fungi might be one of the successful weapons to protect food crops, susceptible to high temperature. These endophytes are known to secrete vital stress responsive secondary metabolites that not only provide resistance to crops against abiotic stresses but also help in promoting plants-growth and yield. Therefore, endophytic fungi were isolated from the leaves and roots of *Adiantum capillus-veneris* L. and their potential were checked for secreting bioactive secondary metabolites and possessing plant-growth promoting potential. A high concentration of phenolics, flavonoids, salicylic acid (SA) and indole 3-acetic acid (IAA) were found in the culture filtrate of isolate AdR-1, thus confirming their growth promoting potential for plants. Later on, phylogenetic exploration and 18S rDNA sequence homology showed that the selected isolatic fungus is *Rhizopus oryzae*. This strain was inoculated to sunflower and soybean seedlings exposed to normal (25°C) and high (40°C) temperature stress in a growth chamber and their potential for thermal stress resistance was compared with control seedlings. Inoculated sunflower and soybean plants had shown low level of abscisic acid (ABA) while, high levels of ascorbic acid oxidase (AAO), catalase (CAT), proline, phenolics, flavonoids, sugars, proteins and lipids were noted. The endophytic fungus was also found stimulatory to chlorophyll content, shoot and root lengths, fresh and dry biomass as compared to un-inoculated plants. These results confirmed the beneficial role of *R. oryzae* for crop plants under thermal stress and suggested their use for sustainable agriculture in the future.

Key words: Endophytic fungi, *Rhizopus oryzae*, Abscisic acid, Thermal stress.

Introduction

Helianthus annuus L. (sunflower) and *Glycine max* L. (soybean), are the two important lipids and protein rich crops, facing heat stress due to global warming. The thermal stress is many fold high in arid, semi-arid and tropical zones of the globe (Fischer & Knutti, 2015). Abiotic stresses including high temperature significantly affect vital physiological processes of agricultural crops that result in the reduction of food quantity and quality. Endophytic fungi are the beneficial asymptomatic partners of plants and new tools that recently used by plant physiologists against abiotic and biotic stresses (Khan *et al*., 2019). Heat stress boosts up transpiration rate and produces physiological drought that causes reduction in crop yield. However, there are differences in crops sensitivity to high temperature stress as maximum value for soybean is 30° C, sunflower 33°C, corn 29°C, maize 36°C and cotton 32°C (Gornall *et al*., 2010). Drought and heat stresses significantly reduce crops capacity to use water efficiently and hence their growth and yield (Stratonovitch & Semenov, 2015). Disturbances in the level of phytohormones were also reported in many plants under thermal stress. Jasmonic acid (JA) and abscisic acid (ABA) increase during high temperature stress while, reduction has been noted in the amount of gibberellin (GA_3) and auxin (Waqas *et al*., 2012). Low level of cytokinin was reported in *Passiflora edulis* (passion fruit) under thermal stress that retarded shoot meristem expansion and flowers format ion (Sobol *et al*., 2014). Endophytic fungi are recognized to synthesize plant-growth stimulating phytohormones including IAA and GAs that restore plant growth and development under abiotic and biotic stresses. Another beneficial role of endophytes for their host plant is enhancement in the absorption of essential minerals like Ca, P, K, Mg and S (Yuan *et al*., 2010). Endophytes also have a role in the improvement of plants' nutritious value like total proteins, carbohydrates and lipids under drought, cold and salt stresses (Ikram *et al*., 2019).

Concentration of reactive oxygen species (ROS) increases by many folds in stressful conditions that results in degradation of cell components leading to premature cell death. But high content of phenolics and proline play a protective role against ROS (Naseem *et al.,* 2018). Endophytic fungi are known to secrete phenolic compounds inside host tissues that cause reduction in ROS concentration synthesized under various stresses. Phytohormones secreted by endophytic fungi including IAA, GAs, JA, SA and ABA along with phenolics, are the stress alleviating tools that help to lower negative effects of different environmental stresses (Khan *et al*., 2020a; Mehmood *et al.,* 2018a). However, adaptive capabilities of agricultural crops like sunflower and soybean to thermal stress, is not well characterized. Here we tried to highlight the potential role of endophytic fungi associated to sunflower and soybean under high temperature stress and their effect on the total nutritive value.

Materials and Methods

The experimental work was performed in the Department of Botany, Abdul Wali Khan University Mardan (AWKUM), Khyber Pakhtunkhwa, Pakistan. Rice seeds (Fakhr-e-Malakand Variety) were obtained from Agricultural Biotechnology Institute (ABI), National Agricultural Research Center (NARC), Islamabad, Pakistan. Rice seedlings were grown in pots having 30 ml of water agar medium (0.8%) in a growth chamber set at day/night cycles: 14 h, 28°C ± 0.3 day and 10 h, 25°C ± 0.3 night, relative humidity was 70%. Sunflower (ICI Hyson 33 Australia) and soybean (Swat 84) varieties were grown in autoclaved sand for two weeks in growth chamber set at 25ºC and 40ºC for 14 h, 28°C ± 0.3 day and 10 h, 25°C ± 0.3 night, relative humidity was 70% and 14 h, 40°C ± 0.3 day and 10 h, 35°C ± 0.3 night, relative humidity was 70% respectively. All experiments were performed in replicates of three.

Isolation of endophytic fungi from *Adiantum capillus-veneris* L.: Endophytic fungi were isolated from *Adiantum capillus-veneris* L. collected from District Swat Khyber Pakhtunkhwa, Pakistan. Plants-sample were scrubbed with tap-water and Tween-80 solution and disinfected with 5% sodium hypochlorite for 5 min. Then samples were cut into 0.5 and 1 cm sections and kept on Hagam media plates (10 pieces/plate) (0.5% glucose, 0.05% KH2- PO4, 0.05% MgSO4·7H2O, 0.05% NH4Cl, 0.1% FeCl3, 80 ppm streptomycin and 1.5% agar; pH 5.6 ± 0.2) (Khan *et al*., 2008). After the emergence, fungal isolations were refined on potato dextrose agar (PDA) media by repeated sub-culturing. Clear isolates were stored at 4°C while, for longer storage PDA (water 1000 ml, potato (Sliced washed unpeeled) 300 g, glucose 20 g and agar 20 g) slants were made (Khan *et al*., 2009). For the analysis of secondary metabolites, fungal isolates were then inoculated to Czapek broth medium (50 ml) Czapek (1% $C_6H_{12}O_6$, 1% Peptone, 0.05% MgSO4·7H2O, 0.05% KCl, 0.001% $FeSO_4.7H_2O$; pH 7.3 ± 0.2) in a shaking incubator set at 30°C for 7 days at 120 rpm. Pellets and supernatants were separated using filter paper.

Screening-bioassay of fungal filtrates on rice plants: Fungal filtrate (100 µl) was applied for their growth stimulating or retarding nature on the tip of rice plantlets at 2 leaf phase grownup in 30 ml of 0.8% water-agar medium in growth chamber for 1 week. Growth attributes (shoot and root lengths, fresh and dry biomass) were noted after 7 days of filtrate application and compared tallied with control plants (Czapek treated and distilled water seedlings) (Ismail *et al*., 2016).

Molecular identification of fungal isolates: Methodology of Khan *et al*., (2008) was followed for fungal DNA isolation, PCR amplification and molecular identification of AdR-1 isolate. ITS-1 and ITS-4 primers were used for the amplification of the internal transcribed regions of 18S rDNA. The obtained sequences were subjected to BLASTn1 software for sequence similarity assessment. Phylogenetic analysis was carried out with Neighbor Joining (NJ) tree via MEGA-7 software.

Inoculation of *Rhizopus oryzae* to sunflower and soybean: Pellet of *R. oryzae* was inoculated to pots (1 mg/100 g of sand) containing 6 seeds of sunflower and soybean both then transferred to growth chambers fixed at 40°C and 25°C for 14 days whereas, supernatant was analyzed for the presence of secondary metabolites. For watering, Hoagland solution was given to the pots at 48 hours interval and growth attributes were analyzed after 2 weeks of endophyte inoculation.

Determination of IAA and SA in culture filtrate of *R. oryzae*: Standardized protocol of Benizri *et al*., (2013) was applied for the analysis of IAA in the culture filtrate of *R. oryzae* (AdR-1). One ml filtrate was added to 2 ml of Salkowski reagent and incubated for half an hour in the dark at room temperature. The color developed, was checked by the Perkin Elmer Lambda 25 spectrophotometer at 540 nm. Different concentrations of IAA i.e. 10, 20, 30, 40, 60, 80 and 100 µg/ml were used for making standard curve.

We used Warrier *et al*., (2013) methodology for the determination of SA in the culture filtrate of AdR-1. A 100 µl of filtrate was added to 2.99 ml of 0.1% freshly formed solution of $FeCl_3$. Appearance of violet color showed the presence of SA because of a complex formed by SA with Fe. Optical density was taken after the color developed at 540 nm. Different SA (Sigma Aldrich) concentrations (100, 200, 300, 400 and 500 µg/ml) were taken to make standard curve.

Analysis of plant growth features: Two weeks old sunflower and soybean seedlings were analyzed for total chlorophyll contents, shoot and root lengths, -fresh and dry biomass of endophyte-associated and –free seedlings.

Analysis of endogenous ABA: Yoon *et al*., (2009) methodology was applied for the determination of ABA concentration in sunflower and soybean plants grownup at 25°C and 40°C. 0.5 g of fresh leaves of both sunflower and soybean were crushed in liquid N_2. A 30 ml mixture of glacial acetic acid and isopropanol (19:1) was added, filtered and then dried using rotary evaporator (Rotary Evaporator 1L/2L R-1001VN/R-1001LN). Then diazomethane was mixed with that combination and examined by GCMS SIM (GC System 6890N Network, via 5973 Network Mass Selective Detector, Agilent Technologies, Palo Alto, CA, USA). The Lab. Base (Thermo Quset, Manchester, UK) Data Analyzer Software was used to examine replies to ions with mass to charge (m/z) values of 162 and 190 for Me-ABA whereas, 166 and 194 for Me-[2H6]-ABA. Abscisic acid ([2H6]-ABA) (Sigma Aldrich) was used as an internal standard.

Determination of proline, flavonoids and phenolics in sunflower and soybean seedlings, and in the culture filtrate of *R. oryzae*: Mervet *et al.*, (2009) protocol was used for the examination of flavonoids in sunflower and soybean plants and, in the culture filtrate of AdR-1. Sunflower and soybean leaves (0.5 g) were crushed in 5 ml ethanol (80%) and kept in a shaking incubator set at 120 rpm for 24 h. Then the sample was spun for 15 min at 10,000 rpm. After spinning, supernatant (250 µl) was mixed with DW (1.25 ml)

and then 5 µl of $NaNO_2$ (5%) was combined with the mixture and incubated for five minutes. A of 10% $AlCl_3.H_2O$ (150 µl) was also added to this mixture and incubated for 6 minutes. In the last, 275 µl of DW and 500µl of NaOH (1M) were combined in test tube and mixed well. Aqueous ethanol (80%) was used as blank. Optical density was taken at 415 nm**.** Different concentrations of quercetin (Sigma Aldrich) (15, 30, 60, 120, 240 and 480 µg/ml) were taken to make standard curve.

Bates *et al*., (1973) methodology was applied for the adetermination of prolin in sunflower and soybean leaves. Sunflower and soybean leaves (0.1 g) were crushed in 4 ml of 3% sulpho-salicylic acid and incubated for 24 hours at 5°C. The mixture was then spun at 3000 rpm for 5 min. Acid ninhydrin (2 ml) and supernatant (2 ml) were mixed and warmed upto 100°C for an hour. Optical density was taken at 520 nm after the addition of 4 ml toluene. Toluene was used as blank. Different concentrations (2, 4, 6, 8 and 10 µg/ml) of proline were used to make standard curve.

Folin-Ciocalteu colorimetric methodology of Cai *et al.*, (2004) was applied for the determination of total phenolics in fungal filtrate, sunflower and soybean. Folin-Ciocalteu reagent (0.5 N) was combined with sample (0.2 ml) and incubated at 25°C for four minutes. For the neutralization, saturated Na_2CO_3 (75 g/L) was combined to this mixture followed by heating at 100°C for one minute. Mixture was incubated for 2 hours in the dark. Optical density was taken at 650 nm. Different concentrations of Gallic acid (100, 200, 300, 500 and 600, 700 and 900 mg/ml) were used to make the standard curve.

Analysis of CAT and AAO in sunflower and soybean plants: CAT analysis in sunflower and soybean plants was done using methodology of Lack (1974). Two g leaves were crushed in of phosphate buffer solution (10 ml) and spun at 10,000 rpm for 5 min and, then supernatant was collected. H_2O_2-phosphate buffer (3 ml) and supernatant (40 µl) were combined. Optical density was taken at 240 nm. H_2O_2-free Phosphate-buffer solution was used as blank. A decrease of 0.05 in the absorbance at 240 nm was calculated as one enzyme unit.

Oberbacher and Vines (1963) methodology was applied for the determination of AAO in sunflower and soybean plants. One g fresh leaves were crushed in 2 ml of phosphate buffer, spun at 3000 rpm for five minutes and then supernatant (100 µl) and substrate solution; 3 ml, (ascorbic acid (8.8 mg) and phosphate buffer (300 ml); PH 5.6), was combined. Optical density was taken at 265 nm for 5 min after every 30 seconds of interval. One AAO unit was calculated by drop in OD by 0.01 per minute.

Determination total proteins, sugars and lipids in sunflower and soybean plants: Lowery *et al*., (1951) protocol was used for the determination of proteins in sunflower and soybean seedlings. 1 g of fresh leaves were used and OD was taken at 650 nm. Different concentrations (20, 40, 60, 80 and 100 µg/ml) of BSA was used to make standard curve. Mohammadkhani and Heidari (2008), methodology was applied for the determination of soluble sugar in soybean and sunflower leaves (0.5 g) and OD was taken at 485 nm. Different concentrations (20, 40, 60, 80 and 100 µg/ml) of glucose (Sigma Aldrich) was used to make the standard curve. Van Handel (1985), methodology was applied for the analysis of lipids in sunflower and soybean seedlings. Sunflower and soybean leaves (0.2 g) were crushed and mixture of H_3PO_4 and Vanillin blue was used as blank. Optical density was taken at 490 nm.

Statistical Analysis

All experimentations were performed in replicates of three. The data were evaluated by one way analysis of variance (ANOVA) and their means were compared with Duncan multiple range test (DMRT) at p=0.05 using SPSS-20.

Results

Isolation of endophytic fungi and their screening bioassay on rice seedlings: A total of 12 different fungal isolates were collected from the leaves and roots of *Adiantum capillus-veneris* L. Growth attributes were noted after one week of filtrate application and compared with Czapek control and DW control treatments (Table 1). Fungal isolate AdR-1 was known to be growth promoter of rice seedlings and selected for further study and molecular identification. The isolate was identified as *Rhizopus oryzae*.

Phylogenetic analysis of isolate AdR-1: DNA of fungus was extracted and sequenced for molecular identification. BLAST result of 28S rDNA sequencing, was analyzed through MEGA 7.0 software, which showed 81% homology with *R. oryzae*. Phylogenetic tree constructed from 11 taxa (1 clone and 10 references) by Neighbor Joining (NJ) method via MEGA 7 package (Fig. 1) confirmed strain identity as *R. oryzae*. The *R. oryzae* (AdR-1) sequence was submitted to NCBI Gene Bank under accession No. MH577052.

Table 1. Effect of *R. oryzae* (AdR-1) filtrate on the growth of rice seedlings.

Growth attributes	Control (DW)	Control (Czk)	*R. oryzae*
Shoot length (cm)	12.7 + 0 0.36^{a}	13.8 + 0.54^{ab}	15.7 + 0.62^{b}
Root length (cm)	6.4 + 0.58^{a}	6.7 + 0.17^{ab}	7.7 + 0.35^{b}
Fresh weight shoot (g)	0.0301 + 0.003^{a}	0.042 + 0.001^{b}	0.05 + 0.001^{b}
Fresh weight root (g)	0.0937 + 0.007^{a}	0.0986 + 0.006^{a}	0.141 + 0.001^{b}
Dry weight shoot (g)	0.0045 + 0.001^{a}	0.0052 + 0.003^{a}	0.0071 + 0.004^{b}
Dry weight root (g)	0.0137 + 0.001^{a}	0.0147 + 0.003^{a}	0.0162 + 0.004^{b}

Screening bioassay of fungal filtrate (100µl) on rice seedlings at two leaves stage, grown in .8% water-agar medium at room temperature. Data are means of 3 replicates with standard error. For each set of treatment, the different letter indicates significant differences at p<0.05 > as estimated by Duncan's Multiple Range Test (DMRT). DW = Distilled water, Czk = Czapek, AdR-1 = Fungal strain isolated from the root of *Adiantum* L.

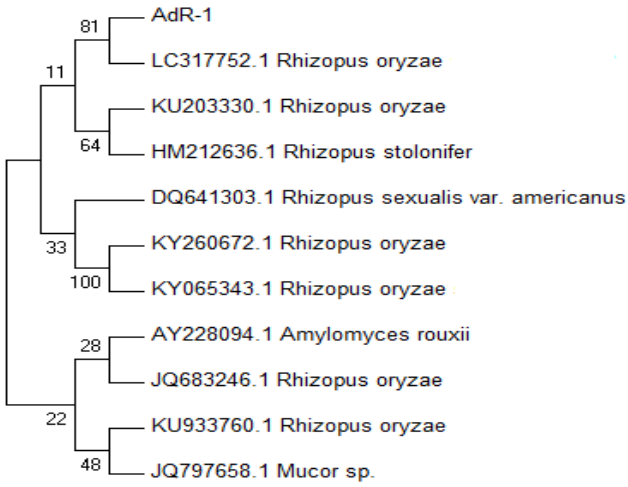

Fig. 1. Phylogenetic consensus tree construction (using 11 taxa, 10 reference and 1 clone) for the identification of fungal isolate AdR-1 using neighbor joining (NJ) method. 81% bootstrap value confirmed isolate AdR-1 as *R. oryzae*.

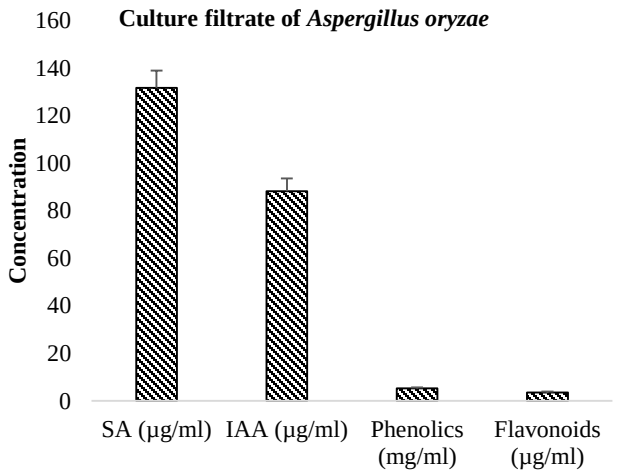

Fig. 2. Important secondary metabolites secreted by *R. oryzae* (AdR-1) in their culture filtrate grown for 1 week in Czapek broth medium in shaking incubator set at 120 rpm at 28ºC. Data are means of 3 replicates with standard error bars.

Analysis of secondary metabolites in *Rhizopus oryzae* filtrate: Different concentrations of secondary metabolites found in fungal filtrates were, SA (131.67 µg/ml), IAA (88.16 µg/ml), phenolics (5.25 mg/ml) and flavonoids (3.59 µg/ml) (Fig. 2).

Endophyte-sunflower and soybean interface: A significant enhancement in all growth features has been recorded in sunflower and soybean seedling co-cultured with *R. oryzae* at 25ºC and 40ºC. Sunflower and soybean inoculated with *R. oryzae* had more chlorophyll content (6.4% and 3.6%), shoot (13% and 18%) and root length (74% and 6.5%), fresh weight shoot (13.7% and 1.6%), fresh weight root (61.7% and 87.9%) and dry weight shoot (11.7% and 2.9%) and dry weight root (0% and 9%) respectively under heat stress compared to non-inoculated seedlings (Tables 2 and 3).

Modulation of endogenous ABA in soybean and sunflower under temperature stress: In both sunflower and soybean seedlings, a significant decrease (76.9% in sunflower and 77% in soybean plants) was observed in the concentration of ABA at heat stress when inoculated with *R. oryzae* as compared to non-inoculated seedlings. Sunflower inoculated with *R. oryzae* had 34 ng/g of ABA while, control plants had 60 ng/g at 40ºC. Similarly, *R. oryzae* treated soybean had 29.9 ng/g while, non-inoculated plants had 262 ng/g of ABA concentration at high temperature. At 25ºC both sunflower and soybean seedlings inoculated with and without *A. oryzae* had no significant differences in the amount of ABA (Fig. 3).

Influence of *R. oryzae* on the endogenous concentrations of flavonoids, proline and phenolics: Total flavonoids, proline and phenolic concentrations of sunflower and soybean inoculated with and without *R. oryzae* at 25ºC and 40ºC, were analyzed via spectrophotometer. At 25ºC, control sunflower seedling had 36 µg/g and *R. oryzae*-associated seedlings had 51.7 µg/g of flavonoids while, at 40ºC *R. oryzae*-associated seedlings had 40 µg/g and *R. oryzae*-free seedlings had 24.6 µg/g of flavonoids. Similarly, *R. oryzae*-associated and -free soybean has 25 µg/g 20.9 µg/g at 25ºC and 61 µg/g 38 µg/g of flavonoids at 40ºC respectively (Fig. 4). Maximum proline concentration (16 µg/g) was found in *R. oryzae*-associated soybean seedling at 40ºC while, control soybean seedling has13 µg/g of proline content. *Rhizopus oryzae*-soybean had 11.5 µg/g while, control seedling had 6.6 µg/g of proline at 25ºC. Sunflower had less content of proline as compared to soybean. *R. oryzae*-associated sunflower seedling had 3 µg/g while, control seedling had 1.8 µg/g of proline at 25ºC. At 40ºC, *R. oryzae*-associated sunflower had 3.7 µg/g while, *R. oryzae*-free sunflower seedlings has 3 µg/g of proline contents (Fig. 4B). Enhanced content of phenolics were found in *R. oryzae*-associated-soybean (8.2 mg/g) at 40ºC as compared to control seedlings (7.8 mg/g). While, at 25ºC, *R. oryzae*-associated-soybean had 3.5 mg/g and non-inoculated plants had 2.8 mg/g of phenolic compounds. Sunflower associated with *R. oryzae* at 25ºC, had 2 mg/g and non-associated plants had 1.3 mg/g of phenolics while, at 40ºC, sunflower inoculated with *R. oryzae* has 5.9 mg/g as compared to non-inoculated seedlings 4 mg/g (Fig. 4C).

Enhancement in the activity of AAO and CAT: A noteworthy proliferation was found in the antioxidants activity of AAO and CAT in sunflower and soybean plants aligned with *R. oryzae* at heat stress. Maximum activity of CAT (1.4 units enzymes/g/30 seconds) was found in soybean aligned with *R. oryzae* at 40ºC while, control seedling had 0.9 units enzymes/g/30 seconds. At 25ºC soybean associated with *R. oryzae* had 0.7 units enzymes/g/30 seconds as compared to endophyte-free seedlings (0.5 units enzymes/g/30 seconds). Similarly, sunflower associated with *R. oryzae* had 0.4 units enzymes/g/30 seconds at 25ºC and 0.8 units enzymes/g/30 seconds at 40ºC while, endophyte-free sunflower had 0.2 units enzymes/g/30 seconds and 0.6 units enzymes/g/30 seconds at 25ºC and 40ºC respectively (Fig. 5).

Table 2. Effect of endophytic fungal strain *R. oryzae* (AdR-1) on the growth of sunflower.

Growth attributes/temperature stress	25°C		40°C	
	Control	*R. oryzae*	Control	*R. oryzae*
Total chlorophyll content (SPAD)	38.4 ± 1.9[a]	44 ± 3.7[a]	39 ± 1.3[a]	41.5 ± 2.4[a]
Shoot length (cm)	20.5 ± 0.4[a]	24.5 ± 1.1[b]	23.7 ± 0.9[ab]	26.8 ± 1.5[b]
Root length (cm)	6± 0.6[a]	8 ± 0.4[ab]	9 ± 0.6[b]	15.7 ± 1.4[c]
Fresh weight shoot (g)	1.22 ± 0.09[a]	1.43 ± 0.25[a]	1.09 ± 0.04[a]	1.24 ± 0.18[a]
Fresh weight root (g)	0.188 ± 0.05[b]	0.361 ± 0.02[c]	0.075 ± 0.007[a]	0.196 ± 0.03[b]
Dry weight shoot (g)	0.040 ± 0.00006[a]	0.081 ± 0.0007[b]	0.085 ± 0.009[b]	0.095 ± 0.0006[b]
Dry weight root (g)	0.014 ± 0.0003[a]	0.04 ± 0.0001[b]	0.024 ± 0.001[a]	0.024 ± 0.007[a]

Note: Effect of endophytic fungus *R. oryzae* (AdR-1) on sunflower seedlings, isolated from *Adiantum capillus-veneris* L. Data are means of 3 replicates with standard error. Different letters are significantly different (p<0.05) as estimated by Duncan's Multiple Range Test (DMRT)

Table 3. Effect of endophytic fungal strain *R. oryzae* (AdR-1) on the growth of soybean.

Growth attributes/temperature stress	25 °C		40 °C	
	Control	*R. oryzae*	Control	*R. oryzae*
Total chlorophyll content (SPAD)	29 ± 2[a]	34.8 ± 1.2[b]	30.7 ± 0.3[ab]	31.8 ± 0.6[ab]
Shoot length (cm)	260 ± 0.9[a]	35 ± 1.7[b]	38.7 ± 1.8[b]	45.7 ± 2.2[c]
Root length (cm)	10 ± 0.6[a]	17.1 ± 2[a]	15.3 ± 3.2[a]	16.3 ± 1.4[a]
Fresh weight shoot (g)	1.138 ± 0.02[b]	1.173 ± 0.28[b]	0.121 ± 0.37[a]	0.123 ± 0.02[a]
Fresh weight root (g)	0.182 ± 0.01[a]	0.166 ± 0.07[a]	0.133 ± 0.02[a]	0.25 ± 0.07[a]
Dry weight shoot (g)	0.079 ± 0.0003[a]	0.078 ± 0.0002[a]	0.135 ± 0.01[b]	0.139 ± 0.01[b]
Dry weight root (g)	0.01 ± 0.0001[a]	0.023 ± 0.0003[b]	0.075 ± 0.002[c]	0.082 ± 0.006[c]

Note: Effect of endophytic fungus *R. oryzae* (AdR-1) on soybean seedlings, isolated from *Adiantum capillus-veneris* L. Data are means of 3 replicates with standard error. Different letters are significantly different (p<0.05) as estimated by Duncan's Multiple Range Test (DMRT)

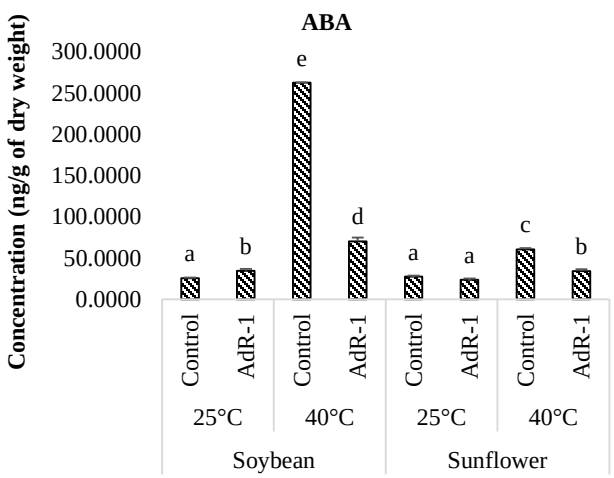

Fig. 3. GC MS analysis of ABA concentration of sunflower and soybean aligned with *R. oryzae* (AdR-1). Data are means of 3 replicates. Similar bars labeled with different letters are significantly different (p<0.05).

Sunflower and soybean aligned with *R. oryzae* had 1 units enzymes/g/30 seconds and 1.7 units enzymes/g/30 seconds at 25°C while, control seedlings had 0.6 units enzymes/g/30 seconds and 1.5 units enzymes/g/30 seconds. At 40°C, *R. oryzae*-associated sunflower and soybean has 3 units enzymes/g/30 seconds and 2.5 units enzymes/g/30 seconds as compared to control sunflower (2.3 units enzymes/g/30 seconds) and soybean (1.8 units enzymes/g/30 seconds) plants (Fig. 5B).

Improvement in host´s nutrients by *R. oryzae* under heat stress: Total proteins, sugars and lipids of *R. oryzae*-associated sunflower and soybean were determined spectrophotometrically. A slight decrease was observed in the total protein content of endophyte-inoculated sunflower (33%), while a small rise was detected in soybean (10.6%) under thermal stress as compared to normal temperature. At 40°C, *R. oryzae*-associated sunflower and soybean had 193 µg/g and 218 µg/g of protein content while, endophyte free sunflower has 125 µg/g and soybean had 163 µg/g of total proteins. At 25°C, *R. oryzae*-associated sunflower and soybean had 258 µg/g and 197 µg/g of protein content while, endophyte free sunflower has 204 µg/g and soybean had 213 µg/g of total proteins (Fig. 6).

At 25°C, *R. oryzae*-aligned sunflower and soybean had 168 µg/g and 208 µg/g of total soluble sugar concentration as compared to non-inoculated sunflower (105 µg/g) and soybean (133 µg/g) while, at high temperature (40°C), endophyte-associated sunflower had 91 µg/g and soybean had 206 µg/g as compared to *R. oryzae*-free sunflower (76 µg/g) and soybean (202 µg/g) seedlings (Fig. 6B). Total lipids of sunflower and soybean were analyzed through spectrophotometer. At 25°C, *R. oryzae*-treated sunflower had 198 µg/g and soybean has 346 µg/g lipids while, control sunflower had 143 µg/g and soybean had 281 µg/g of lipids. At 40°C, *R. oryzae*-associated sunflower had 168 µg/g and soybean has 255 µg/g of lipids as compared to endophyte-free sunflower 112 µg/g and soybean 187 µg/g (Fig. 6C).

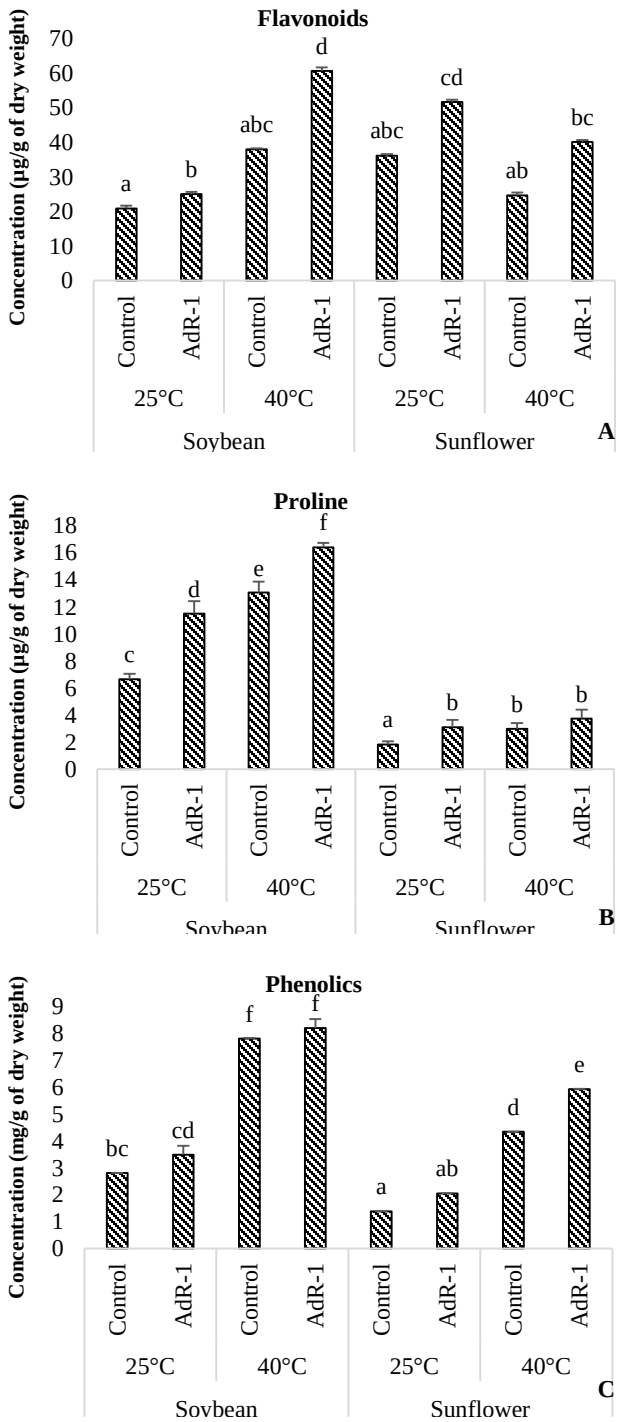

Fig. 4. Analysis of (A) flavonoids, (B) proline and (C) phenolics of sunflower and soybean associated with *R. oryzae.* Data are means of 3 replicates with standard error bars labeled with different letters are significantly different (p<0.05).

Discussion

Endophytic fungi live inside the plant tissues as symbiotic partners with no noticeable signs of disease (Rodriguez *et al*., 2008) and that have a role in secreting vital secondary metabolites for their host plant (Mehmood *et al.,* 2019). We investigated heat-stress alleviating potential of plant growth promoting endophytic fungus *Rhizopus oryzae* for sunflower and soybean at 40ºC. Rice plantlets were used for the initial screening of *R. oryzae* for their growth promoting potential because of its quick and easy response to plant growth promoting hormones including IAA and GAs (Hamayun *et al*., 2015). Like other endophytic fungi, *R. oryzae* also secretes vital secondary metabolites in their culture filtrate like IAA, SA, flavonoids and phenolics. Indole 3-acetic acid is observed to have critical role against abiotic and biotic stress responses via regulation and transcription of several anti-stress genes (Fahad *et al*., 2015). Several physiological and developmental processes are considered to be orchestrated by IAA (Mehmood *et al.,* 2018b; Teale *et al*., 2006). In *Arabidopsis thaliana*, IAA promotes hypocotyl growth during thermal stress Thermal stress represses the expression of IAA biosynthesis genes i.e. YUCCA that leads to abortion of pollen grains development (Sakata *et al*., 2010). Salicylic acid has a role as defense signaling chemical against biotic and abiotic stress stimuli. It also takes part in plant growth and development, flower induction, ethylene biosynthesis, regulation of respiration and stomatal behavior (Waqas *et al*., 2015). Flavonoids (Agati *et al*., 2012) and phenolics (Suzuki *et al*., 2014) function as strong antioxidants against ROS generated during high temperature stress. IAA and GAs were previously analyzed in the culture filtrate of some endophytic fungi comprising *Fusarium oxysporum, Aspergillus niger*, *Paecilomyces formosus, Penicillium funiculosum* (Ali *et al.,* 2018; Deng & Cao, 2017).

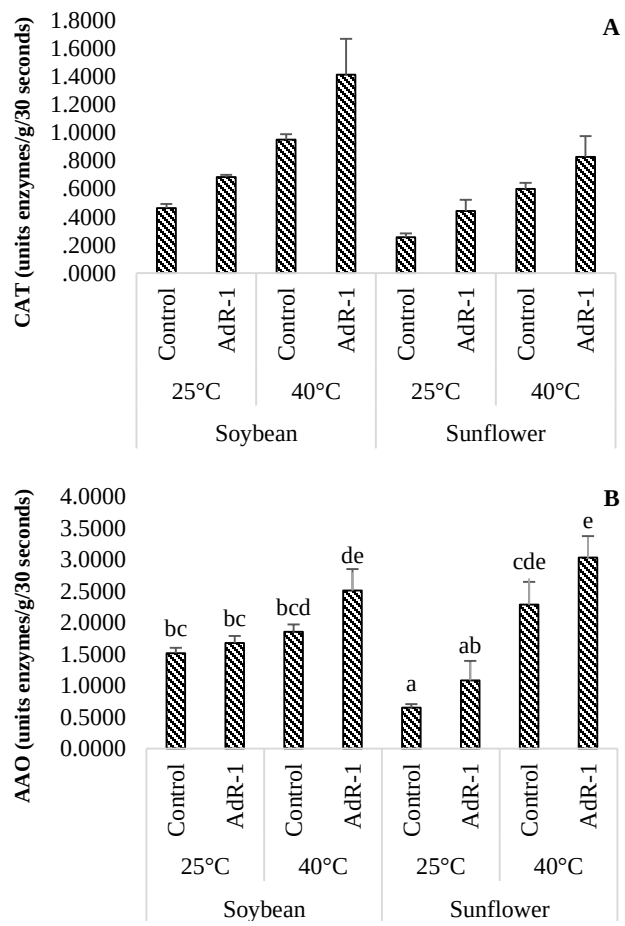

Fig. 5. Analysis of CAT (A) and AAO (B) concentration of sunflower and soybean seedlings treated with *R. oryzae* (AdR-1). Data are means of 3 replicates with standard error bars labeled with different letters are significantly different (p<0.05).

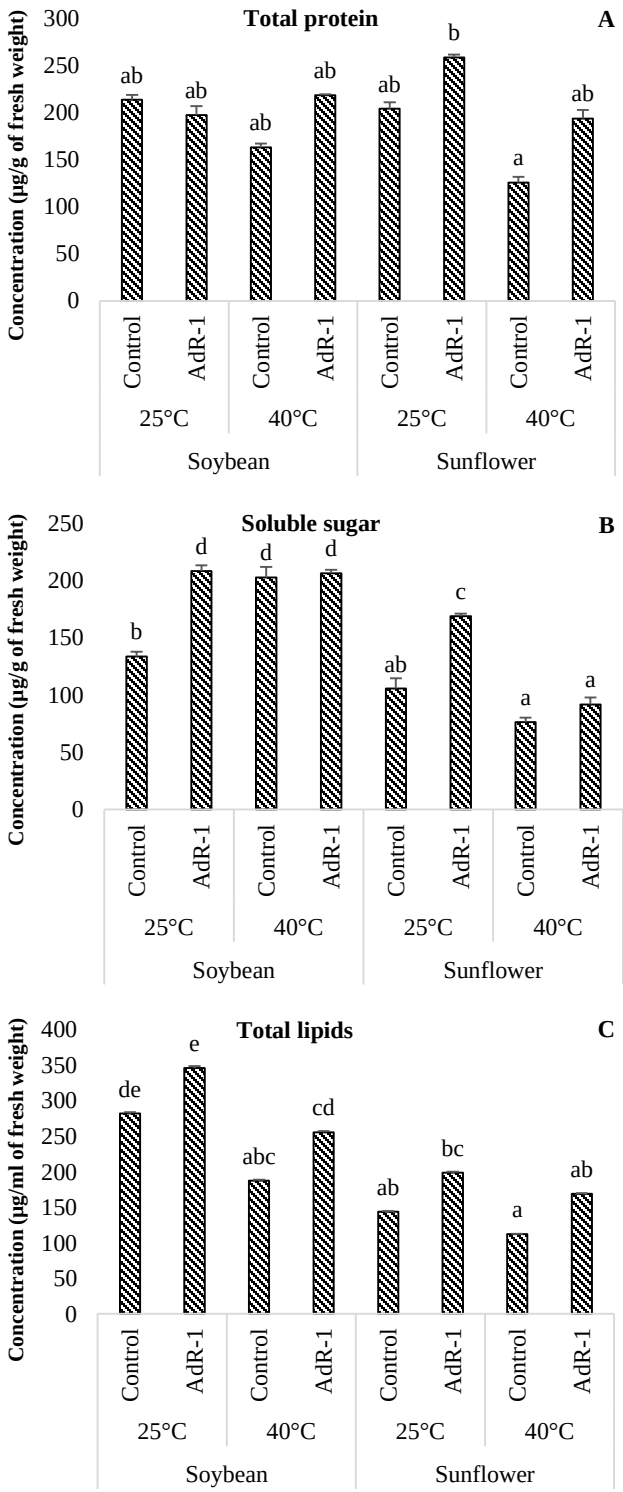

Fig. 6. Analysis of total proteins (A), soluble sugars (B) and lipids (C) of sunflower and soybean seedlings treated with *R. oryzae* (AdR-1). Data are means of 3 replicates with standard error bars labeled with different letters are significantly different (p<0.05).

Proline, an amino acid, occurs in higher plants in retort to different abiotic stresses as osmolyte, free radicals scavenger and as buffer for cellular-redox potential (Balal *et al.,* 2017). It also has role in mitigating cytoplasmic acidosis, maintenance of correct ratio of NADP+/NADPH that might be compatible with plant cellular metabolism (Hare & Cress, 1997) and protein compatible hydro-trope (Srinivas & Balasubramanian, 1995). On overcoming of stress conditions, rapid breakdown of accumulated proline release sufficient amount of strong reducing agents that enhance process of ATP synthesis and mitochondrial oxidative phosphorylation as well as repair damages induced by stress (Hare & Cress, 1998). Moreover, proline also help in expression of those abiotic stress-responsive-genes which have proline-responsive-elements (e.g. PRE, ACTCAT) in their promoter regions (Khan *et al*., 2020b). High concentration of proline in *R. oryzae*-associated sunflower and soybean as compared to non-inoculated seedlings at 40ºC, clearly indicated thermal alleviating capability of endophytic fungi. Our result confirmed previous work that endophytic fungi caused enhancement in phenolics content in tall fescue plant under stress (Zhou *et al*., 2003). An enzyme phospho-lypase-D, Ca and ABA, are known to be responsible as signaling chemicals that regulate P5CS gene, responsible for proline biosynthesis (Thiery *et al*., 2004). Sunflower and soybean aligned with endophytic fungus *R. oryzae* have low level of ABA as compared to non-inoculated seedlings of sunflower and soybean at thermal stress of 40ºC. Low level of ABA in endophyte-associated seedlings determines beneficial role of endophytic fungi because accumulation of ABA is noted in plants subjected to heat stress (Badshah *et al.,* 2012; Raghavendra *et al*., 2010).

ROS are unstable free radicals generated by all plants during normal metabolic reactions where they function as mediator of cell division, differentiation, progression and defense against biotic and abiotic stresses while, their enhanced concentration under stress is the leading cause of premature aging and apoptosis (Krishnamurthy & Wadhwani, 2012). All organisms are known to have their own antioxidant-defense-system (ADS), responsible for deactivation of ROS by reducing their vitality or distressing their chain of oxidizing reactions, earlier than they become injurious to the cell (Alici & Arabaci, 2012). Different abiotic and biotic stresses cause enhancement in the synthesis of H_2O_2 that harm vital cell components but at the same time plants have defensive antioxidant enzymatic system in the form of CAT, AAO and SOD (Al-Saffar et al., 2006). CAT and AAO target and degrade excess of ROS including singlet oxygen, superoxides, hydroxyl radicals and H_2O_2 (Mhamdi *et al*., 2010). CAT has multi genes family in plants that have role in encoding important proteins. These proteins help in regulation, localization and expression of stress responsive genes in a cell under different stresses (Su *et al*., 2014). Study on AAO suggested that it had role in balancing the concentration of membrane bound antioxidant α-tocopherol in a low amount. It is also known that AAO help in cell wall reorganization by performing vital role as cofactor for peptidyl-lysyl and peptidyl-prolyl hydroxylases that speed up biosynthesis of hydroxyl-lysine and hydroxyl-proline. Later on, Hydroxy-proline is transformed to Hydroxyproline-rich-glycoproteins (HRGPs). HRGPs play vital structural and supportive role for plant cell wall under different environmental stresses including heat stress (Conklin, 2014). High amount of AAO and CAT in *R. oryzae*- associated soybean and sunflower as compared to un-inoculated plantlets under

high temperature stress, suggested that *R. oryzae* helped their host plant in mitigating thermal stress. In a similar study on ryegrass, Bonnet *et al.*, (2000) noted enhanced concentration of AAO in endophyte associated plants. It has also been reported previosly that endophytic fungi like *Aspergillus flavus* has protective role for host plant in reducing thermal toxicity (Rodriguez *et al.*, 2012). Sugars play an important role for plants like chief energy source, storage compounds, and precursor for carbon containing compounds and polymers synthesis. Several sugars including glucose, galactose, raffinose and sucrose are known to gather in plant tissues as a result of abiotic stresses (Khan *et al.*, 2018). In our study, we observed an enhanced level of total soluble sugar, protein and lipids in *R. oryzae*-associated sunflower and soybean compared un-inoculated plants, suggesting the beneficial role of endophytes for their host plants.

Conclusion

Current work was an endeavor to know the role of endophytic fungus *R. oryzae* for sunflower and soybean under heat stress. These oily crops are facing continuous thermal stress in their growing areas because of global warming. Global warming is a world's combined potent problem that significantly reduce crops quality and quantity. Reduction in the severity of thermal stress responses by endophytic fungus *R. oryzae*, inoculated to sunflower and soybean suggested its use as bio-fertilizer and thermal stress alleviating agent in the future for sustainable agriculture.

References

Agati, G., E. Azzarello, S. Pollastri and M. Tattini. 2012. Flavonoids as antioxidants in plants: Location and functional significance. *Plant. Sci*., 196: 67-76.

Ali, S., N. Khan, F. Nouroz, S. Erum, W. Nasim and M.A. Shahid. 2018. In vitro effects of GA 3 on morphogenesis of CIP potato explants and acclimatization of plantlets in field. *In Vitro Cell. Dev. Biol. Plant*., 54: 104-111.

Alici, E.H. and G. Arabaci. 2016. Determination of SOD, POD, PPO and CAT enzyme activities in the *Rumex obustifolius* L. *Ann. Res. Rev. Biol*., 11(3): 1-7.

Al-Saffar, N.M., H. Troy, de.M.A. Ramirez, L.E. Jackson, B. Madhu and J.R. Griffiths. 2006. Noninvasive magnetic resonance spectroscopic pharmacodynamic markers of the choline kinase inhibitor MN58b in human carcinoma models. *Cancer. Res*., 66: 427-434.

Badshah, H., R.A. Qureshi, J. Khan, F. Ullah, S. Fahad, F. Ullah, A.M. Khan, I. Hussain and N. Khan. 2012. Pharmacological screening of *Morchella esculenta* (L.) Pers., *Calvatia gigantea* (Batsch ex Pers.) Lloyd and Astraeus hygrometricus Pers., mushroom collected from South Waziristan (FATA.). *J. Med. Plants Res.*, 6: 1853-1859.

Balal, R.M., M.A. Shahid, M.M. Javaid, Z. Iqbal, G.D. Liu, L. Zotarelli and N. Khan. 2017. Chitosan alleviates phytotoxicity caused by boron through augmented polyamine metabolism and antioxidant activities and reduced boron concentration in *Cucumis sativus* L. *Acta Physio. Plant.*, 39: 31.

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

Benizri, E., A. Courtade, C. Picard and A. Guckert. 1998. Role of maize root exudates in the production of auxins by *Pseudomonas fluorescens* M.3.1. *Soi. Biol. Biochem*., 30: 1481-1484.

Bonnet, M., O. Camares and P. Veisseire. 2000. Effect of zinc and influence of *Acremonium lolii* on growth parameters, chlorophyll *a* fluorescence and antioxidant enzyme activities of ryegrass (*Lolium perenne* L. cv Apollo). *J. Exp. Bot*., 51: 945-953.

Cai, Y., Q. Luo, M. Sun and H. Corke. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci*., 74: 2157-2184.

Conklin, P.L. 2001. Recent advances in the role and biosynthesis of ascorbic acid in plants. *Plant. Cell Environ*., 24(4): 383-394.

Deng, Z. and L. Cao. 2017. Fungal endophytes and their interactions with plants in phytoremediation: a review. *Chemosphere*, 168: 1100-1106.

Fahad, S., S. Hussain, A. Matloob, F.A. Khan, A. Khaliq, S. Saud, S. Hassan, D. Shan, F. Khan, N. Ullah and M. Faiq. 2015. Phytohormones and plant responses to salinity stress: a review. *Plant Growth Reg.*, 75: 391-404.

Fischer, E.M. and R. Knutti. 2015. Anthropogenic contribution to global occurrence of heavy-precipitation and high-temperature extremes. *Nat. Clim. Chang*., 5: 560-564.

Gornall, J., R. Betts, E. Burke, R. Clark, J. Camp, K. Willett and A. Wlstshire. 2010. Implications of climate change for agricultural productivity in the early twenty-first century. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.,* 365: 2973-2989.

Hamayun, M., A. Hussain, S.A. Khan, M. Irshad, A.L. Khan, M. Waqas, R. Shahzad, A. Iqbal, N. Ullah, G. Rehman and H.Y. Kim. 2015. Kinetin modulates physio-hormonal attributes and isoflavone contents of Soybean grown under salinity stress. *Front. Plant. Sci*., 6: 377.

Hare, H. and W.A. Cress. 1997. Metabolic implications of stress-induced proline accumulation in plants. *Plant. Growth. Reg*., 21: 79-102.

Hare, P.D., W.A. Cress and J.V. Staden. 1998. Dissecting the roles of osmolyte accumulation during stress. *Plant. Cell. Environ*., 21: 535-553.

Ikram, M., N. Ali, G. Jan, F. Guljan and N. Khan. 2019. Endophytic fungal diversity and their interaction with plants for agriculture sustainability under stressful condition. *Recent Pat. Food Nutr. Agric.,* 10: DOI: 10.2174/2212798410666190612130139.

Ismail, M. Hamayun, A. Sayyed, I.U. Din, H. Gul and A. Hussain. 2016. Gibberellin and indole acetic acid production capacity of endophytic fungi isolated from *Zea mays* L. *Int. J. Biosci*., 8(3): 35-43.

Khan, N., A. Bano and A. Babar. 2020b. Impacts of plant growth promoters and plant growth regulators on rainfed agriculture. *PloS One.*, 15: e0231426.

Khan, N., A. Bano, M.A. Rahman, B. Rathinasabapathi and M.A. Babar. 2019. UPLC-HRMS-based untargeted metabolic profiling reveals changes in chickpea (*Cicer arietinum*) metabolome following long-term drought stress. *Plant Cell Environ.,* 42: 115-132.

Khan, N., A. Bano, M.A. Shahid, W. Nasim and M.A. Babar. 2018. Interaction between PGPR and PGR for water conservation and plant growth attributes under drought condition. *Biologia.*, 73: 1083-1098.

Khan, N., A. Bano, S. Ali and M.A. Babar. 2020a. Crosstalk amongst phytohormones from planta and PGPR under biotic and abiotic stresses. *Plant Growth Reg.*, 90: 1-15.

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

Khan, S.A., M. Hamayun, H.Y. Kim, H.J. Yoon, J.C. Seo and Y.S. Choo. 2009. A new strain of *Arthrinium phaeospermum* isolated from *Carex kobomugi* Ohwi is capable of gibberellin production. *Biotechnol. Lett*., 31: 283-287.

Krishnamurthy, P. and A.M. Wadhwani. 2012. Antioxidant enzymes and human health. *Antioxidant Enzyme*., 3-18.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., 193(1): 265-75.

Luck, H. 1974. Methods in enzymatic analysis. Academic Press, New York, 885.

Mehmood, A., A. Hussain, M. Irshad, M. Hamayun, A. Iqbal and N. Khan. 2019. In vitro production of IAA by endophytic fungus *Aspergillus awamori* and its growth promoting activities in *Zea mays*. *Symbiosis.,* 77: 225-235.

Mehmood, A., A. Hussain, M. Irshad, N. Khan, M. Hamayun, Ismail, S.G. Afridi and I.J. Lee. 2018b. IAA and flavonoids modulates the association between maize roots and phytostimulant endophytic *Aspergillus fumigatus* greenish. *J. Plant Interact.,* 13: 532-542.

Mehmood, A., N. Khan, M. Irshad and M. Hamayun. 2018a. IAA Producing Endopytic Fungus *Fusariun oxysporum* wlw Colonize Maize Roots and Promoted Maize Growth Under Hydroponic Condition. *Eur. Exp. Biol.,* 8: 24.

Mervat, M., M.E. Far and H.A. Taie. 2009. Antioxidant activities, total anthocyanins, phenolics and flavonoids contents of some sweet potato genotypes under stress of different concentrations of sucrose and sorbitol. *Aust. J. Bas. App. Sci*., 3(4): 3609-3616.

Mhamdi, A., G. Queval, S. Chaouch, S. Vanderauwera and F. Van-Breusegemet. 2010. Catalase functions in plants: a focus on *Arabidopsis* mutants as stress-mimic models. *J. Exp. Bot*., 61: 4197-4220.

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

Naseem, H., M. Ahsan, M.A. Shahid and N. Khan. 2018. Exopolysaccharides producing rhizobacteria and their role in plant growth and drought tolerance. *J. Basic Microbiol.*, 58: 1009-1022.

Oberabacher, M.F. and H.M. Vines. 1963. Spectrophotometric assay of ascorbic acid oxidase. *Nature.*, 197: 1203-1204.

Raghavendra, A.S., V.K. Gonugunta, C. Alexander and G. Erwin. 2010. ABA perception and signaling. *Trends Plant. Sci.,* 15: 395-401.

Rodriguez, R. and R. Redman. 2008. More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *J. Exp. Bot*., 59: 1109-1114.

Rodriguez, R.J., C.J. Woodward and R.S. Redman. 2012. Fungal influence on plant tolerance to stress. In biocomplexity of plant-fungal interactions; southworth, D., Ed.; Wiley-Blackwell: Oxford, UK.

Sakata, T., T. Oshino, S. Miura, M. Tomabechi, Y. Tsunaga, N. Higashitani, Y. Miyazawa, H. Takahashi, M. Watanabe and A. Higashitani. 2010. Auxins reverse plant male sterility caused by high temperatures. *Proc. Natl. Acad. Sci.,* 107: 8569-8574.

Sobol, S., N. Chayut, N. Nave, D. Kafle, M. Hegele and R. Kaminetsky. 2014. Genetic variation in yield under hot ambient temperatures spotlights a role for cytokinin in protection of developing floral primordia. *Plant. Cell. Environ*., 37: 643-657.

Srinivas, V. and D. Balasubramanian. 1995. Proline is a protein-compatible hydrotrope. *Langmuir*, 11: 2830-2833.

Stratonovitch, P. and M.A. Semenov. 2015. Heat tolerance around flowering in wheat identified as a key trait for increased yield potential in Europe under climate change. *J. Exp. Bot*., 66(12): 3599-609.

Su, Y., J. Guo, H. Ling, S. Chen and S. Wang. 2014. Isolation of a novel peroxisomal catalase gene from sugarcan, which is responsive tobiotic and abiotic stresses. *Plos One*, 9(1): e84426.

Suzuki, N., R.M. Rivero, V. Shulaev, E. Blumwald and R. Mittler. 2014. Abiotic and biotic stress combinations. *New. Phytol*., 203: 32-43.

Teale, W.D., I.A. Paponov and K. Palme. 2006. Auxin in action: signalling, transport and the reverse plant male sterility caused by high temperatures. *Proc. Natl. Acad. Sci. USA*., 107: 8569-8574.

Thiery, L., A. Leprence, D. Lefebvre, M.A. Ghars, E. Debarbeuixand and A. Savoure. 2004. Phospholipase D is negative regulator of proline biosynthesis in *Arabidopsis thaliana*. *J. Boil. Chem*., 279: 14812-14818.

Van Handel, E. 1985. Rapid determination of total lipids in mosquitoes. *J. Amer. Mosq. Control. Assoc*., 1(3): 302-4.

Waqas, M., A.L. Khan, M. Hamayun, R. Shahzad, S. Kang and J. Kim. 2015. Endophytic fungi promote plant growth and mitigate the adverse effects of stem rot an example of *Penicillium citrinum* and *Aspergillus terreus*. *J. Plant. Interact*., 10: 280-287.

Waqas, M., A.L. Khan, M. Kamran, M. Hamayun, S.M. Kang, Y.H. Kim and I.J. Lee. 2012. Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. *Molecules*, 17: 10754-10773.

Warrier, R.R., M. Paul and M.V. Vineetha. 2013. Estimation of salicylic acid in *Eucalyptus* leaves using spectrophotometric methods. *Gen. Plant. Physiol*., 3(1-2): 90-97.

Yoon, J.Y., M. Hamayun, S.K. Lee and I.J. Lee. 2009. Methyl jasmonate alleviated salinity stress in soybean. *J. Crop. Sci. Biotechnol*., 2: 63-68.

Yuan, Z.L., C.L. Zhang and F.C. Lin. 2010. Role of diverse non-systemic fungal endophytes in plant performance and response to stress: progress and approaches. *J. Plant Growth. Regul*., 29: 116-126.

Zhou, F., Y.B. Gao and W.J. Ma. 2003. Effects of phosphorus deficiency on growth of perennial rye grass fungal endophyte symbiont and phenolic content in root. *Plant. Physiol. Comm.,* 39: 321-325.

(Received for publication 20 December 2018)