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# An endophytic fungus *Aspergillus violaceofuscus* can be used as heat stress adaptive tool for *Glycine max* L. and *Helianthus annuus* L.

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## Summary

High temperature is one of the leading threats to the plants that severely affects crop quality as well as quantity. Endophytic fungi might be a new tool to safeguard crops against the perilous effects of global warming. In this context, we isolated a thermal stress mitigating endophytic fungus from the fern *Dryopteris filix* L. The phylogenetic study and 18S rRNA sequence similarity confirmed the potential strain as *Aspergillus violaceofuscus*. The culture filtrate of *A. violaceofuscus* exhibited higher concentration of secondary metabolites that enhanced the total chlorophyll content, plant height and biomass of sunflower and soybean seedlings under heat stress. Conversely, the *A. violaceofuscus* associated plants achieved low levels of reactive oxygen species, abscisic acid, catalase, ascorbic acid oxidase, proline and an overall improved the nutritional value. The current study suggests that *A. violaceofuscus* can be used as heat stress adaptive tool in crops to achieve sustainable agriculture.

**Keywords:** endophytic fungi, global warming, *Aspergillus violaceofuscus, Dryopteris filix*., antioxidants, heat stress

## Introduction

Rising temperature is one of the long lasting and challenging threats faced by the globe especially in arid, semiarid, and tropical zones, where mean annual temperature is already high. Being a sessile nature, plants are always exposed to such unfriendly situation that lead to a high concentration of reactive oxygen species (ROS) including hydrogen peroxide ($H_2O_2$), hydroxyl radical (OH), singlet oxygen ($^1O_2$) and superoxide ($O_2^{-1}$) (Ismail et al., 2019). Thermal stress not only accelerates the generation of ROS that cause premature cell death, but also results in salinity by enhancing evaporation from the soil (Ismail et al., 2018). ROS contain free electrons that cause apoptosis. At the same time biological membranes have local antioxidant system that immediately detoxify these ROS species earlier than their act to deter the cell (Quan et al., 2008). Catalase (CAT), ascorbic acid oxidase (AAO), glutathione reductase (GR), peroxidase (POD) and superoxide dismutase (SOD) are part of the specific enzymatic antioxidant system while tocopherol, ascorbic acid and some secondary metabolites constitute the non-enzymatic antioxidant system of plants (Hamayun et al., 2015; Hamayun et al., 2017). Among the different antioxidants, AAO and CAT especially target the oxidizing chain reactions or energy source of ROS (Mhamdi et al., 2010). CAT also plays a role in the expression of stress-receptive-genes (SRGs) by synthesizing specific proteins that regulate their expression (Su et al., 2014).

Phytohormones, including salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA), have a role in signaling against biotic and abiotic stresses (Bilal et al., 2018; Ismail et al., 2019). ABA has a defensive role in the amelioration of drought and heat stress by regulating opening and closing of stomata as well as restoration of plant growth and development (Yoon et al., 2009). JA helps in the biosynthesis of defensive proteins, secondary metabolites, as well as control of senescence, pollen development and root growth (Lorenzo et al., 2004). SA has a role in respiration, growth, development, ethylene synthesis, stomatal response, and biotic stresses like pathogens and insects (Waqas et al., 2012).

Biosynthesis of phenolics and proline are accelerated during abiotic stresses and play a role as ROS scavengers, osmolytes and in buffering of cellular redox reactions (Nusrat et al., 2019). Breakdown of proline after stress generates large amounts of reducing agents that are then used for the biosynthesis of ATP in mitochondrial oxidative phosphorylation. Later on, these ATP molecules are used in stress recovery and repairing of damages (Ashraf and Foolad, 2007).

Endophytic fungi, such as *Aspergillus japonicas* (Ismail et al., 2018), *Aspergillus flavus* (Ismail et al., 2019), *Trichoderma reesei* (Muhammad et al., 2019) and *Penicillium roqueforti* (Ikram et al., 2018) are known to be present in most plants with no visible symptom of harm, and have a role in refurbishment of the host plant during biotic and abiotic stresses. These endophytes can reduce ailment severity, prove resistance, accelerate mineral absorption and enhance biomass synthesis (Mehmood et al., 2019a; Mehmood et al., 2019b; Rodriguez et al., 2012). High temperature stress can severely affect the viability of plant species leading to high yield losses. Plant species can counter the heat stress in several ways, including accumulation of chemicals. Calcium, kinases, reactive oxygen species, carbohydrate, transcription factors, gene expression regulation, and plant hormones signaling pathways can play accessible part in up-regulating the key genes liable to confront the high temperature stress (Zahid et al., 2016). Endophytes are known to secrete important secondary metabolites, like phytohormones (IAA, GA, JA and SA), proline, phenolics and flavonoids in their cultural filtrates and perhaps in host's tissue (Ali et al., 2019; Bilal et al., 2018). This means that endophytic metabolites can help plants under stress to up-regulate the genes that lead to secretion of endogenous hormones in order to ensure normal growth and development (Bilal et al., 2018). It has been demonstrated in the past that non-endophytic fungi containing plants are more vulnerable to intense light, drought, salinity as well as heat stress in comparison to endophyte-hosting plants (Ismail et al., 2019; Ismail et al., 2018; Kang et al., 2019).

The present work was therefore designed to isolate potent endophytic strain(s) from the medicinal plant species *D. filix* and explore its role in high temperature stress resistance in *H. annuus* and *G. max*.

## Materials and methods

### Plant collection and isolation of endophytic fungi

A wild medicinal plant *D. filix*, was collected from dry rocky sunside mountain of District Swat, Tehsil Kabal (DD COORDINATES: 34.7833302 72.2833322; DMS COORDINATES: 34°46'59.99" N

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72°16'60.00" E), Khyber Pakhtunkhwa, Pakistan in the month of July. The plants were carried to plant microbes' interaction (PMI) laboratory. *D. filix* was selected for the isolation of endophytes because many of the medicinal plant species, we have explored previously served as a pool of potent endophytes. Therefore, the idea was focused to explore the diversity of fungal endophytes in *D. filix* and study their role in heat-stress amelioration.

To isolate endophytic fungi, the standardized protocol of KHAN et al. (2008) was followed. Hagam media was used to isolate endophytic fungi, refined on Potato Dextrose Agar (PDA) media, and then shifted to fridge at 4 °C. Endophytic fungi were grown in Czapek medium (50 ml) in a shaking-incubator for seven (7) days set at 28 °C at 120 revolutions per minute (rpm). Secondary metabolites were collected and analyzed via PerkinElmer Lambda 25 double beam spectrophotometer (ISMAIL et al., 2019). Seven cultures were isolated from *D. filix* roots and three cultures were isolated from *D. filix* leaves.

**Fungal filtrate screening on *Oryza sativa* L. seedlings**

Filtrates (100 µl) of endophytic fungi were applied on top of *O. sativa* (variety: Fakhr-e Malakand, provided by the Agriculture Research Station, Mingora, Swat, Pakistan) for examination of their potential to stimulate growth or hinder secondary metabolites at two leaf phase, grown up in 0.8% (v/w) water-agar media in a growth-chamber for one week (day; 14 hrs 28 °C ± 0.3; night; 10 hrs 25 °C ± 0.3 and 70% relative humidity). Total chlorophyll contents, root-shoot lengths, and fresh and dry weight of fungal filtrate treated seedlings (experimental) was compared with distilled water (DW) and Czapek (Czk) treated seedlings (control) after 1 week of incubation.

**Identification of fungal isolate**

KHAN et al. (2008) methodology was applied for the molecular identification of endophytic fungi, using internal transcribed regions (ITS) of 18S rRNA via ITS1 (forward) (5' - TCC GTA GGT GAA CCT GCG G - 3') and ITS4 (reverse) (5' - TCC TCC GCT TAT TGA TAT GC - 3'). The resultant nucleotide order was exposed to BLASTn1 software for sequence similarity estimate. Phylogenetic tree was constructed with NJ (Neighbor Joining) via MEGA-7 Software.

***Aspergillus violaceofuscus* inoculation to *H. annuus* and *G. max***

*Aspergillus violaceofuscus* was grown in a 250 ml conical flask having Czapek broth of 50 ml and transferred to shaking-incubator set at 28 °C for 1 week at 120 rpm. Filter paper was used to separate pellets and supernatant. One mg fresh endophytic fungi bio-mass per 100 g of autoclaved sand, was applied to pot comprising *H. annuus* (variety Hysun-33) and *G. max* (variety Swat-84) seeds (9 seeds/pot), then transferred for 14 days into growth chambers fixed at normal temperature at 25 °C and high temperature at 40 °C. Supernatants were checked for the existence of vital secondary metabolites. Ten milliliter Hoagland Solution (half strength) was given to plants at two days interval. Growth attributes were assessed after 14 days of cultivation in growth chambers (MISRA and DWIVEDI, 2004). Experiment was conducted in triplicates.

**Analysis of Indole acetic acid and salicylic acid in the culture filtrate of *A. violaceofuscus***

BENIZRI et al. (1998) methodology was followed to investigation IAA in the filtrate of *A. violaceofuscus*. One ml fungal filtrate and 2 ml of Salkowski reagent were mixed and then kept in the dark for half an hour at 25 °C. The optical density was observed at 540 nm via PerkinElmer Lambda 25 spectrophotometer. Various concentrations of IAA were applied to build a standard curve (10, 20, 30, 40, 60, 80, and 100 µg/ml).

The protocol of WARRIER et al. (2013) was used for the determination of SA. Fungal filtrate (100 µl) and 0.1% Iron chloride solution (2.99 ml) was mixed and OD was checked at wavelength of 540 nm after appearing violet color. Known concentration (40, 60, 80, 100 and 120 µg/ml) of SA was made to draw a standard curve.

**Endogenous investigation of ABA in *H. annuus* and *G. max* seedlings**

The methodology of YOON et al. (2009) was used to investigate ABA concentration in *H. annuus* and *G. max* seedlings. Soybean and sunflower's fresh leaves (0.5 g) were crushed in liquid $N_2$ and mixed with 2 ml of glacial acetic acid (GAA, 28.5 ml) and isopropanol (1.5 ml) mixture. Then via rotary evaporator, the mixture was filtered and dehydrated. Then we added diazomethane to this mixture and examined via GC MS SIM (6890N setup GC Scheme furnished with 5973 System Mass Selective Detector; Agilent Technologies, Palo Alto, CA, USA). The Lab Base, Thermo Quset, Manchester, UK, Data System Software (DSS) was used to monitor retorts to ions with m/z standards of 190 and 162 for Me-ABA and 194 and 166 for Me-[2H6]-ABA. ABA ([2H6]-ABA) was applied as standard.

**Investigations of antioxidants in *H. annuus* and *G. max***

Concentration of CAT in the seedlings of *H. annuus* and *G. max* was determined using the protocol of LUCK (1974). Fresh leaves of sunflower and soybean (2 g each) were ground in 10 ml of phosphate buffer and then spun at 10,000 rpm for 5 minutes. 40 µl of supernatant was mixed with 3 ml of $H_2O_2$-phosphate buffer. Optical density was measured at a wavelength of 240 nm. $H_3PO_4$ buffer was used as blank. The quantity of enzyme needed to lower OD by 0.05 per gram of plant biomass at 240 nm was considered as one unit. OBERBACHER and VINES (1963) methodology was followed to calculate AAO concentration in *H. annuus* and *G. max* 0.1 g fresh leaves of *H. annuus* and *G. max* were crushed in 2 ml of $H_3PO_4$ buffer and spun for 5 minutes at 3000 rpm. Then supernatant (100 µl) and substrate solution (3 ml of 0.0088 g of ascorbic acid (AA) in 0.3 L of $H_3PO_4$ buffer, pH was 5.6) were combined while, OD was taken at 265 nm after every 30 seconds till five minutes.

**Total flavonoids, proline and phenolics concentrations in culture filtrate (CF) of A. *violaceofuscus*, *H. annuus* and *G. max***

Methodology of CAI et al. (2004) was applied to investigate total phenolics in CF of A. *violaceofuscus*, *H. annuus* and *G. max*. Different concentrations of gallic acid (Sigma Aldrich; 100, 200, 300, 500, 600, 700, and 900 mg/ml) were made to draw a standard curve. BATES et al. (1973) procedure was used with minor modifications, to analyze total proline. Various concentrations of proline (Sigma Aldrich; 2, 4, 6, 8, and 10 µg/ml) was used to plot standard curve. Optical density was noted at 520 nm. Flavonoids were analyzed via EL FAR and TAIE (2009) protocol. Different grades of quercetin solution (Sigma Aldrich; 15, 30, 60, 120, 240, and 480 µg/ml) was applied to make standard curve. Optical density at 415 nm was recorded.

**Total proteins, soluble sugars and lipids in *H. annuus* and *G. max* seedlings**

Proteins were determined in *H. annuus* and *G. max* seedlings using methodology of LOWRY et al. (1951). Different concentrations of BSA (Sigma Aldrich; 20, 40, 60, 80, and 100 µg/ml) was applied to draw a standard curve. Absorbance was measured at 650 nm. VAN HANDEL (1985) methodology was followed for the analysis of total lipids. Various concentrations of pure canola oil (10, 40, 70, 100, 130, and 160 µg/ml) was used to draw a standard curve and absorbance

was measured at 490 nm. MOHAMMADKHANI and HEIDARI (2008) protocol was applied to determine soluble sugar in *H. annuus* and *G. max* leaves. Different concentrations of glucose (Sigma Aldrich; 20, 40, 60, 80, and 100 µg/ml) were taken to plot a standard curve while, 485 nm wavelength was used to record the value of absorbance.

**Statistical analysis**

Experiments were conducted in triplicates (3 culture flasks/fungus, 3 pots per measurement and extracts (6 plants/pot). ANOVA was applied to analyze the data. Means of all values were equated by DMRT (Duncan Multiple Range Test) at $p < 0.05$, via SPSS-20 (SPSS Inch., Chicago, IL, USA).

## Results

**Fungal isolation and their screening on *Oryza sativa* seedlings**

A total of ten endophytic isolates, seven from *D. filix* roots and three from *D. filix* leaves (Supplementary Fig. S1), were isolated and initially screened on *O. sativa* seedlings for growth stimulating or hindering potentials (Supplementary Fig. S2). Fungal endophytes were first isolated on plates containing Hagam media and then for purification, grown on PDA media plates. Purification was done on the basis of morphological variations. For filtrate collection, endophytic isolates were cultured in Czapek broth for one week. Growth features (root-shoot length, root-shoot fresh and dry biomass) were recorded after one week of filtrate application on *O. sativa* plantlets at two-leaves stage and are presented in Tab. 1 and 2.

On basis of the most notable changes compared to the controls, the fungal isolate DryL-1 from *D. filix* leaves was chosen for further analysis.

**Molecular identification of fungal isolate DryL-1**

Genomic DNA was extracted fresh mycelium of fungal isolate using ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5' -TCC TCC GCT TAT TGA TAT GC-3'). BLAST search database was used to compare nucleotide sequence of our isolate with the ITS regions of allied fungi. Our nucleotide sequence of 18S rDNA showed 49% resemblance with *A. violaceofuscus*. The phylogenetic harmony tree was prepared from 11 (1 cloned and 10 references) via Neighbor Joining (NJ) technique using MEGA 7 software (Fig. 1). Results of sequence homology and phylogenetic examination proposed that DryL-1 as *A. violaceofuscus*. The sequence was submitted to the gene bank under accession number MH577055.

**Exogenous secretion of secondary metabolites by *A. violaceofuscus* in their culture filtrate**

Flavonoids, phenolics, SA and IAA were analyzed in the CF of *A. violaceofuscus*. Various concentrations of secondary metabolites were flavonoids (2 µg/ml), phenolics (6.6 mg/ml), IAA (34.9 µg/ml) and SA (89 µg/ml) (Fig. 2).

**Tab. 1:** Influence of *A. violaceofuscus* CF on the growth features of rice plants. The isolate DryL-1 marked in bold was chosen for further analysis.

| Isolate | SL (cm) | RL (cm) | FWS (g) | FWR(g) | DWS (g) | DWR (g) | Chl (SPAD) |
|---|---|---|---|---|---|---|---|
| Control DW | $12.83^{ab}$±0.4 | $3.66^{a}$±0.5 | $.030^{b}$±0.003 | $.088^{c}$±0.003 | $.0047^{ab}$±0.001 | $.014^{ab}$±0.001 | $19.33^{a}$±0.88 |
| Control Czk | $13.91^{ab}$±0.5 | $3.83^{a}$±0.1 | $.034^{bcd}$±0.002 | $.092^{c}$±0.002 | $.0067^{abc}$±0.0003 | $.013^{a}$±0.0003 | $22.00^{ab}$±0.57 |
| **DryL-1** | **$15.50^{b}$±0.2** | **$3.83^{a}$±0.9** | **$.058^{e}$±0.0007** | **$.124^{d}$±0.02** | **$.0087^{c}$±0.0004** | **$.018^{b}$±0.0005** | **$24.00^{a}$±1.73** |
| DryL-2 | $13.11^{ab}$±0.5 | $4.16^{ab}$±1.3 | $.050^{cde}$±0.007 | $.101^{cd}$±0.008 | $.0073^{abc}$±0.0003 | $.014^{a}$±0.001 | $22.33^{ab}$±1.20 |
| DryL-21 | $12.66^{ab}$±0.8 | $5.00^{abc}$±0.6 | $.011^{a}$±0.001 | $.056^{b}$±0.00 | $.0053^{abc}$±0.0003 | $.015^{ab}$±0.0006 | $20.66^{ab}$±0.88 |
| DryR-1 | $12.00^{a}$±1.0 | $5.16^{abc}$±0.6 | $.010^{a}$±0.00 | $.010^{a}$±.01 | $.0057^{abc}$±0.001 | $.014^{ab}$±0.001 | $21.66^{ab}$±0.88 |
| DryR-2 | $13.16^{ab}$±0.6 | $6.00^{bcd}$±0.3 | $.031^{bc}$±0.004 | $.091^{c}$±0.00 | $.0047^{ab}$±0.0006 | $.014^{a}$±0.001 | $21.00^{ab}$±1.73 |
| DryR-16 | $13.06^{ab}$±0.9 | $6.25^{cd}$±0.4 | $.031^{bc}$±0.001 | $.110^{cd}$±.012 | $.0040^{a}$±0.00 | $.014^{ab}$±0.002 | $22.00^{ab}$±1.52 |
| DryR-20 | $12.70^{ab}$±1.8 | $6.50^{cd}$±0.5 | $.050^{cde}$±0.014 | $.049^{b}$±0.001 | $.0083^{bc}$±0.001 | $.016^{ab}$±0.001 | $23.00^{ab}$±1.52 |
| DryR-21 | $12.43^{a}$±0.8 | $6.66^{cd}$±0.1 | $.051^{de}$±0.007 | $.061^{b}$±0.004 | $.0080^{bc}$±0.002 | $.016^{ab}$±0.001 | $20.33^{ab}$±0.88 |
| DryR-26 | $13.06^{ab}$±0.7 | $6.83^{cd}$±0.1 | $.035^{bcd}$±0.004 | $.043^{b}$±0.004 | $.0070^{abc}$±0.001 | $.017^{ab}$±0.001 | $22.66^{ab}$±1.45 |
| DryR-30 | $12.76^{ab}$±0.7 | $7.80^{d}$±0.6 | $.042^{bcde}$±0.002 | $.056^{b}$±0.004 | $.0077^{abc}$±0.0003 | $.016^{ab}$±0.001 | $22.66^{ab}$±1.20 |

Data are means of triplicates with standard error bars. Different letters are significantly different ($p < 0.05$) as estimated by Duncan's Multiple Range Test (DMRT). SL = shoot length, RL = root length, FWS = fresh weight shoot, FWR = fresh weight root, DWS = dry weight shoot, DWR = dry weight root, Chl = chlorophyll.

**Tab. 2:** Influence of *A. violaceofuscus* on the growth attributes of *G. max*.

| Growth attributes/ temperature stress | 25 °C | | 40 °C | |
|---|---|---|---|---|
| | Control | *A. violaceofuscus* | Control | *A. violaceofuscus* |
| Total Chlorophyll Content (SPAD) | $29.03+1.98^{a}$ | $30.5+0.76^{ab}$ | $30.73+0.26^{ab}$ | $34.27+1.23^{b}$ |
| SL (cm) | $26+0.93^{a}$ | $36.5+0.65^{a}$ | $38.67+1.76^{b}$ | $46+0.58^{c}$ |
| RL (cm) | $10+0.58^{a}$ | $17+2.06^{b}$ | $16.33+3.18^{b}$ | $17+0.58^{b}$ |
| FWS (g) | $1.138+0.02^{ab}$ | $1.502+0.05^{b}$ | $0.821+0.37^{a}$ | $1.29+0.03^{ab}$ |
| FWR (g) | $0.182+0.01^{ab}$ | $0.223+0.02^{b}$ | $0.133+0.02^{a}$ | $0.405+0.03^{c}$ |
| DWS (g) | $0.079+0.0003^{a}$ | $0.084+0.001^{a}$ | $0.135+0.011^{b}$ | $0.155+0.015^{b}$ |
| DWR (g) | $0.01+0.0001^{a}$ | $0.015+0.0002^{a}$ | $0.075+o.002^{b}$ | $0.12+0.01^{c}$ |

Data are means of triplicates with standard error bars. Different letters are significantly different ($p < 0.05$) as estimated by Duncan's Multiple Range Test (DMRT). DW = distilled water, Czk = Czapek medium, SL = shoot length, RL = root length, FWS = fresh weight shoot, FWR = fresh weight root, DWS = dry weight shoot, DWR = dry weight root.

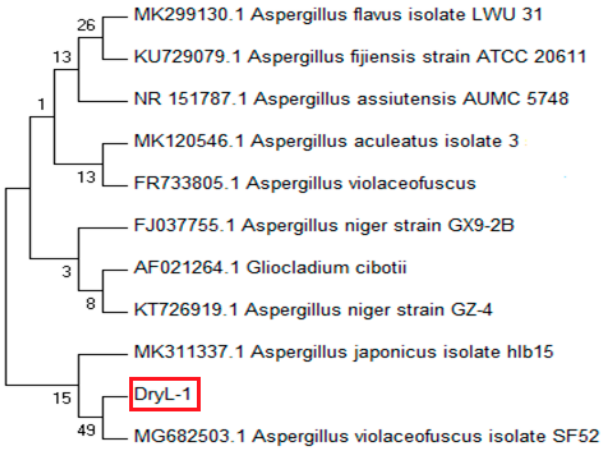


**Fig. 1:** Phylogenetic harmony tree construction (11 taxa, 10 reference and 1 clone) for the identification of fungal isolate DryL-1 using neighbor joining (NJ) method. 49% bootstrap value confirmed isolate DryL-1 as *Aspergillus violaceofuscus*.

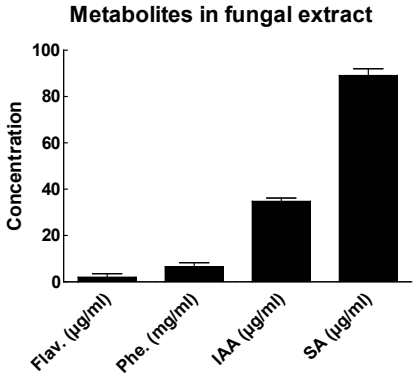


**Fig. 2:** Exogenous production of flavonoids, phenolics, IAA and SA by *A. violaceofuscus* in their CF cultured in Czapek broth for one week using shaking incubator (120 rpm at 28 °C). Data are means of triplicates with standard error bars.

### *A. violaceofuscus* assisted growth promotion of *H. annuus* and *G. max* under heat stress

A significant increase was noted in different growth attributes including shoot-root lengths, fresh and dry weights of shoot and root, and chlorophyll content of both *A. violaceofuscus*-inoculated *G. max* and *H. annuus* at 25 °C and 40 °C as matched with non-inoculated seedlings (Tab. 2 and 3). Higher chlorophyll content, shoot-root lengths, shoot-root fresh and dry weights were found in *G. max* and *H. annuus* seedlings at room temperature (25 °C) than high temperature stress (40 °C) (Tab. 2 and 3).

### Intonation in the endogenous flavonoids, proline and phenolics in A. *violaceofuscus*-inoculated *H. annuus* and *G. max*

Vital secondary metabolites like flavonoids, proline and phenolics were evaluated in *A. violaceofuscus*-inoculated *G. max* and *H. annuus* at 25 °C and 40 °C in a growth chamber. A 22.9% rise was observed in flavonoids content in *A. violaceofuscus*-inoculated *G. max* at 40 °C as related to control plantlets, while at normal temperature the increase was 65.5% in experimental *G. max* seedlings. Similarly, endophyte-aligned *H. annuus* seedlings at 40 °C have just 5% surplus flavonoids as related to the control plantlets, while at 25 °C both control and experimental *H. annuus* seedlings have similar concentrations of flavonoids. An increase was noted in the flavonoids content in *G. max* seedlings at heat stress, while a slight decline was observed in *H. annuus* seedlings as related to normal (25 °C) temperature (Fig. 3A and 3B). Endophyte-associated *G. max* has 60% and 6% more phenolics at 25 °C and 40 °C, respectively as related to non-inoculated plants, while *A. violaceofuscus*-inoculated *H. annuus* has 35.7% at 25 °C and 15% at 40 °C more phenolics as related to non-associated seedlings. (Fig. 3C and 3D). A slight fall was also analyzed in proline concentrations in *A. violaceofuscus*-inoculated *G. max* and *H. annuus* as related to control seedlings. A significant decrease in the proline content was found in *A. violaceofuscus*-aligned *G. max* (49% at normal temperature) and (41% at high temperature) and *H. annuus* (130% at normal temperature) and (68% at high temperature) as compared to control plants (Fig. 3E and 3F).

### Decline in AAO and CAT concentrations

We found a significant decline in CAT and AAO activity in *A. violaceofuscus*-aligned *G. max* and *H. annuus* seedlings as related to endophyte-free seedlings. A reduction was noted in the concentration of CAT in *A. violaceofuscus*-inoculateded *G. max* and *H. annuus* at 25 °C and 40 °C as related to non-inoculated *G. max* and *H. annuus* seedlings (Fig. 4A and 4B). Furthermore, endophyte associated *G. max* had 1.41 enzyme unit/g of leaves and *H. annuus* had 0.75 enzyme unit/g leaves AAO at normal temperature (25 °C) as compared to control *G. max* 01.8 enzyme unit/g leaves and *H. annuus* 1 enzyme unit/g leaves. At high temperature, *A. violaceofuscus*-aligned *G. max* had 1.7 enzyme unit/g of tissue while, *H. annuus* had 2.6 units of enzyme unit/g of tissue as related to non-aligned *G. max* 2.3 enzyme unit/g leaves and *H. annuus* 3 enzyme unit/g leaves (Fig. 4C and 4D).

### Decline in ABA concentration in *H. annuus* and *G. max* plantlets

A substantial decline was observed in the contents of total ABA in *A. violaceofuscus*-associated *G. max* and *H. annuus* plants as related

**Tab. 3:** Influence of *A. violaceofuscus* on the growth attributes of *H. annuus*.

| Growth attributes/ temperature stress | 25 °C | | 40 °C | |
|---|---|---|---|---|
| | Control | *A. violaceofuscus* | Control | *A. violaceofuscus* |
| Total Chlorophyll Content (SPAD) | 38.4+1.9$^{a}$ | 39.8+4$^{a}$ | 37.5+0.4$^{a}$ | 39+1.3$^{a}$ |
| SL (cm) | 20.5+0.4$^{a}$ | 23+1.2$^{ab}$ | 23.7+0.8$^{b}$ | 29+0.6$^{c}$ |
| RL (cm) | 6+0.6$^{a}$ | 13+0.2$^{c}$ | 9+0.6$^{b}$ | 12.3+0.4$^{c}$ |
| FWS (g) | 1.219+0.09$^{bc}$ | 1.404+0.08$^{c}$ | 1.095+0.04$^{ab}$ | 0.871+0.06$^{a}$ |
| FWR (g) | 0.188+0.05$^{a}$ | 0.58+0.16$^{b}$ | 0.075+0.007$^{a}$ | 0.148+0.01$^{a}$ |
| DWS (g) | 0.04+0.00006$^{a}$ | 0.053+0.0007$^{a}$ | 0.085+0.009$^{b}$ | 0.091+0.012$^{b}$ |
| DWR (g) | 0.014+0.0003$^{a}$ | 0.036+0.0008$^{c}$ | 0.024+0.001$^{b}$ | 0.047+0.005$^{d}$ |

Data are means of triplicates with standard error bars. Different letters are significantly different ($p < 0.05$) as estimated by Duncan's Multiple Range Test (DMRT). DW = distilled water, Czk = Czapek medium, SL = shoot length, RL = root length, FWS = fresh weight shoot, FWR = fresh weight root, DWS = dry weight shoot, DWR = dry weight root.

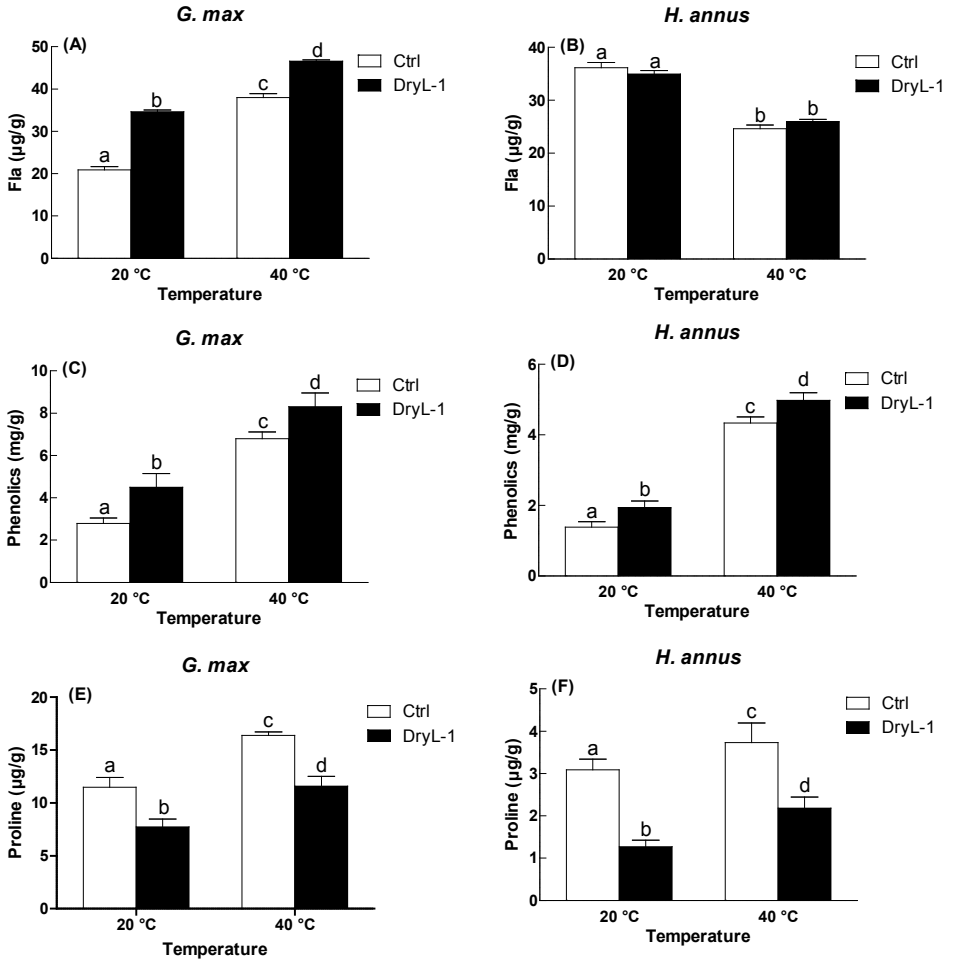


**Fig. 3:** Influence of *A. violaceofuscus* on the concentrations of flavonoids (A, B), phenolics (C, D) and proline (E, F), in *G. max* and *H. annuus* plants grown at normal (25 °C) and high (40 °C) temperatures. Data are means of triplicate with standard error bars. Different letters are significantly different ($p < 0.05$) as estimated by Duncan's Multiple Range Test (DMRT).

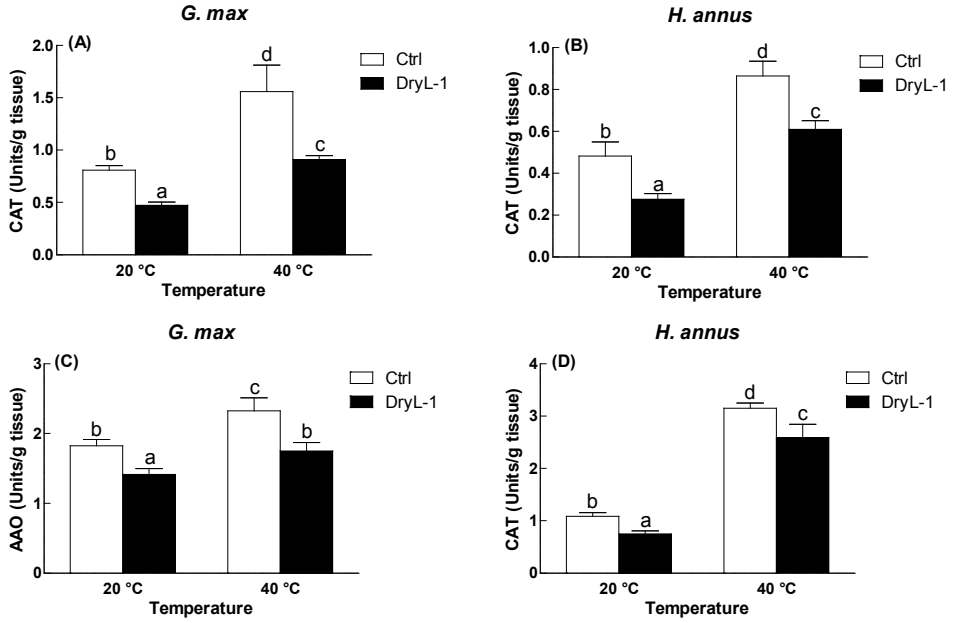


**Fig. 4:** Influence of *A. violaceofuscus* on the concentrations of AAO (A, B) and CAT (C, D) in *G. max* and *H. annuus* plants grown at normal (25 °C) and high (40 °C) temperatures. Data are means of triplicate with standard error bars. Different letters are significantly different ($p < 0.05$) as estimated by Duncan's Multiple Range Test (DMRT).

to non-associated seedlings at 40 °C. *A. violaceofuscus*-inoculated *G. max* has 25.6 ng/g and non-inoculated plants have 28.7 ng/g of ABA concentration at normal temperature, while at thermal stress, endophyte-aligned *G. max* has 42 ng/g while, control plants had 262 ng/g of ABA contents. Similarly, *A. violaceofuscus*-inoculated *H. annuus* has 14.7 ng/g and non-inoculated plants has 27 ng/g at normal temperature while, at thermal stress, endophyte-associated *H. annuus* has 32 ng/g and non-associated *H. annuus* had 60 ng/g of ABA contents (Fig. 5A and 5B).

**Improvement in total nutritious assets of *H. annuus* and *G. max***

The nutritious value of both *G. max* and *H. annuus* was analyzed by determining the contents of proteins, sugars, and lipids. A noteworthy rise was observed in the protein content of *A. violaceofuscus*-inoculated *G. max* (237 µg/g at 25 °C and 189 µg/g at 40 °C) and *H. annuus* (232 µg/g at 25 °C and 147 µg/g at 40 °C) as compared endophyte-free *G. max* (213 µg/g at 25 °C and 163 µg/g at 40 °C) and *H. annuus* (204 µg/g at 25 °C and 125 µg/g at 40 °C) (Fig. 6A and 6B). Similarly, *A. violaceofuscus*-inoculated *G. max* has 180 µg/g while, non-inoculated had 133 µg/g of soluble sugar at normal temperature and, endophyte-aligned *G. max* had 262 µg/g endophyte-free soybean has 202 µg/g at 40 °C. Likewise, *A. violaceofuscus*-associated *H. annuus* had 86 µg/g whereas, non-associated seedlings had 105 µg/g of soluble-sugars at normal temperature (25 °C) and, endophyte-aligned *H. annuus* has 116 µg/g endophyte-free *G. max* has 76 µg/g at 40 °C. (Fig. 6C and 6D). A significant escalation was also found in lipids content in *A. violaceofuscus*-aligned *H. annuus* and *G. max* as related to non-inoculated *G. max* and *H. annuus* plantlets at normal temperature as well as heat stress (Fig. 6E and 6F).

## Discussion

The majority of plants are well-known to have symbiotic relationship with endophytes (Hamayun et al., 2017; Mehmood et al., 2019b). Most of these symbionts synthesize a huge amount of important secondary metabolites, comprising alkaloids, flavonoids and phenolics having importance in agriculture (Bilal et al., 2018; Hamayun et al., 2015). Endophytic fungi help in improving overall growth and development as well as provide immunity against various environmental constrains (Hussain et al., 2018; Muhammad et al., 2019). Therefore, wild medicinal plant species *D. filix* was selected for the isolation of endophytes and then checked for plant growth promotion or inhibition potentials under high temperature stress. *D. filix* was chosen because many of the medicinal plant species, we have explored previously served as a pool of potent endophytes (Ikram et al., 2018; Ismail et al., 2019; Mehmood et al., 2019a; Muhammad et al., 2019). Czapek filtrates of endophytic fungi were primarily evaluated on *O. sativa* plantlets (Fakhr-e-Malakand) for growth encouraging or discouraging potentials. Rice seedlings were chosen because of their easy and quick responses to growth promoting phytohormones, including IAA released by endophytes (Hamayun et al., 2015).

Filtrate of *Aspergillus violaceofuscus* meaningfully boosted rice growth-attributes similar to that of previously tested *Aspergillus japonicus* (Ismail et al., 2018) and *Aspergillus flavus* (Ismail et al., 2019). However, the effect of *A. violaceofuscus* on rice plant shoot and root lengths, and shoot and root dry weights were similar to that of *A. japonicas* (Ismail et al., 2018), but greater than *A. flavis* (Ismail et al., 2019). These results suggest that the endophytic fungi *A. violaceofuscus* has the capacity to improve plant-growth. This argument can also be supported by the results pertinent to the presence of IAA in the culture filtrate of *A. violaceofuscus*. Besides, endophytes are also known to share mutualistic genes with host plants that improve plants overall performance (Rodriguez et al., 2009). Persistent environmental constrains, including salinity, drought and thermal stresses cause hastening of apoptosis, premature cell death and growth inhibition (Iqbal and Ashraf, 2013).

In the present work, we found a positive effect in the overall growth features of sunflower and soybean associated with *A. violaceofuscus*. *A. violaceofuscus*-associated *H. annuus* and *G. max* had more shoot and root lengths, chlorophyll content and biomass as related to non-associated seedlings. Similar observations have been observed in the past studies, where *A. japonicas* (Ismail et al., 2018) and *A. flavis* (Ismail et al., 2019) have also improved the growth parameters of the host plant as compared to their respective controls under high temperature stress. The increase in growth attributes of endophyte-associated *H. annuus* and *G. max* might be due to acceleration in the rate of photosynthesis (Ismail et al., 2019; Ismail et al., 2018). Our study confirm the result of Sun et al. (2010) that *Piriformospora indica* reduces chlorophyll degradation of their host plant under drought stress and sustain the photosynthetic rate. Endophytes improve plants growth and development via secreting IAA and GA in their host tissues (Bilal et al., 2018). This suggests that our endophytic fungus *A. violaceofuscus* boosts the growth parameters of *H. annuus* and *G. max* in normal as well as heat stress conditions as we confirmed the presence of IAA in their CF.

Moreover, *A. violaceofuscus* also enriched the nutritional value of *H. annuus* and *G. max* by enhancing total lipids, proteins and sugars contents at normal and heat stress conditions as related to endophytes-free seedlings. This enrichment in the nutritional quality of sunflower and soybean under heat stress might be due to endophytic fungi that might assist their host plant's to absorb optimal quantities of minerals present in rhizosphere (Ismail et al., 2018). In a similar study, *A. japonicas* (Ismail et al., 2018) and *A. flavis* (Ismail et al., 2019) have also improved the nutritional status of the *H. annuus* and *G. max*. Moreover, the effect of *A. violaceofuscus* was more pronounced on the total lipids, proteins and sugars contents of the *H. annuus* and *G. max* under heat stress as compared to the previously

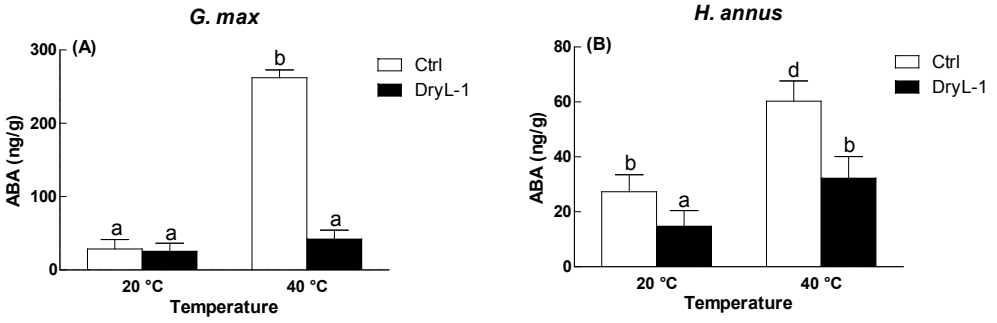


**Fig. 5:** Analysis of ABA quantification via GC MS in *G. max* (A) and *H. annuus* (B) plants grown at normal (25 °C) and high (40 °C) temperatures with and without *A. violaceofuscus*. Data are means of triplicate with standard error bars. Different letters are significantly different ($p < 0.05$) as estimated by Duncan's Multiple Range Test (DMRT).

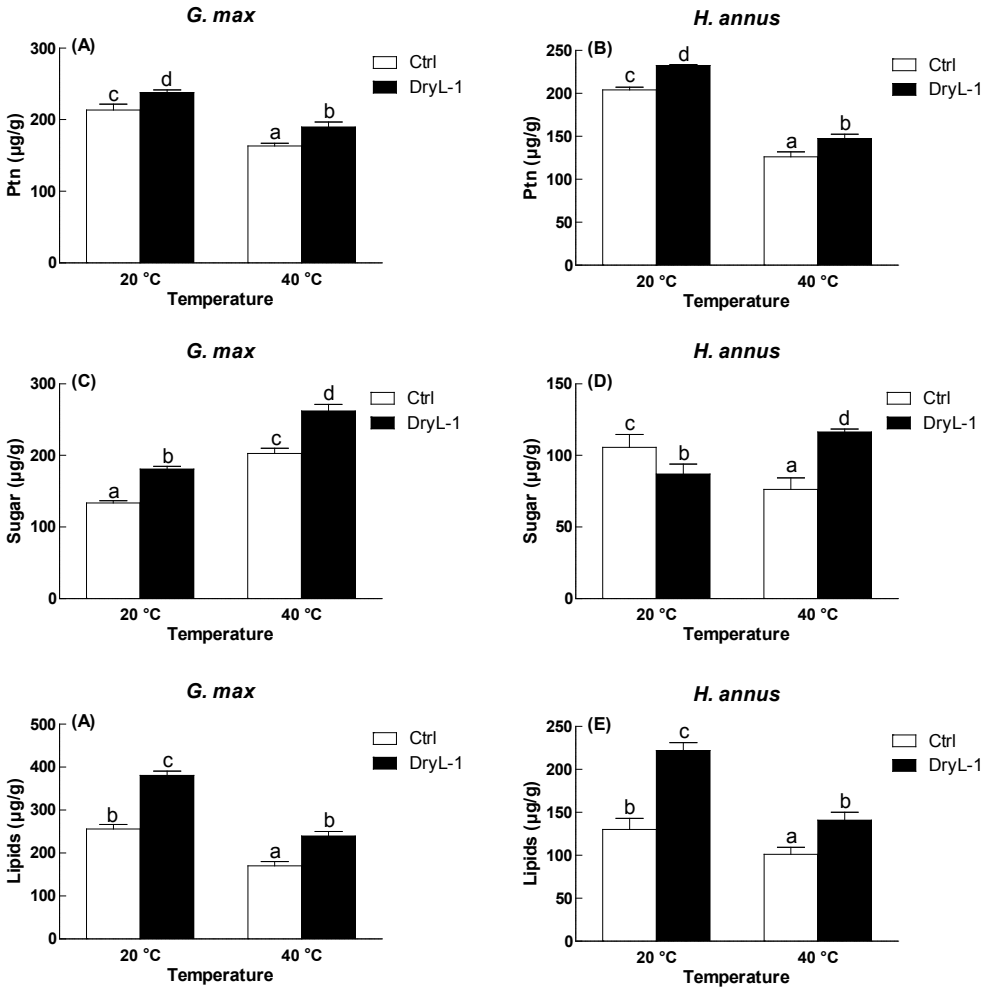


**Fig. 6:** Influence of *A. violaceofuscus* on the concentrations of protein (A, B) and sugar (C, D) and lipids (E, F) in *G. max* and *H. annuus* plants grown at normal (25 °C) and high (40 °C) temperatures. Data are means of triplicate with standard error bars. Different letters are significantly different ($p < 0.05$) as estimated by Duncan's Multiple Range Test (DMRT).

studied endophytes (Ismail et al., 2019; Ismail et al., 2018).

Abscisic acid, a plant hormone, play vital role as signaling compound for plants against a biotic stress. It is known that ABA biosynthetic genes are up regulated during high temperature stress; while at the same time down regulation occur in the genes responsible for catabolism of ABA (Toh et al., 2008). A significant increase occur in ABA concentration during different environmental constrains, including thermal stress (Ismail et al., 2019). In our study, we found a significant decrease in the amount of ABA in *A. violaceofuscus*-associated *H. annuus* and *G. max* subjected to heat stress as compared to control seedlings, representing the ameliorating role of endophytic fungi for their host plants against thermal stress. Similar trends have been observed in previous studies, where *A. japonicas* (Ismail et al., 2018) and *A. flavis* (Ismail et al., 2019) has eased the effect of heat stress in the *H. annuus* and *G. max*. Additionally, the production of ABA was lower in the *A. violaceofuscus* associated-*H. annuus* and *G. max* as compared to the *A. japonicas* (Ismail et al., 2018) and *A. flavis* (Ismail et al., 2019) associated plants under heat stress.

Antioxidants, including flavonoids, proline, phenolics, CAT and AAO are known to reduce ROS toxicity in plants exposed to stressful conditions (Ismail et al., 2019; Ismail et al., 2018). Moderate amount of ROS is essential for cell growth, development and signaling, while their high levels than the threshold value is extremely toxic to plants causing premature cell death. Flavonoids have free radicals scavenging potential and their high concentration in *A. violaceofuscus*-associated *H. annuus* and *G. max* as related to non-associated plantlets, confirmed the ameliorative role of endophytes against heat stress. Plants also gather high amount of proline in their tissues as a protecting agent against cellular redox, a forager against free radicals and as an osmolyte in response to various environmental constrains (Hamayun et al., 2017; Nusrat et al., 2019). In the present work, we found low level of proline in endophyte-associated *H. annuus* and *G. max* as related to endophyte-free seedlings under thermal stress. This confirmed the protective role of endophytic fungi in host plants, while accumulation of high content of proline in control plants proved their role during thermal stress.

Phenolics are a class of natural aromatic secondary metabolites with a role in herbivore deterrence and as antioxidants. Higher plants accumulate different types of phenolics against various stresses as rice accumulates sakuranetin (7-methylated flavanone) against pathogens and ultraviolet light (Park et al., 2013), *N-p*-Coumaroylserotonin (CouSer), *N*-feruyolserotonin (FerSer) and *N*-feruloyltryptamine (FerTrp) against pathogenic fungi (blast fungus and *Bipolaris oryzae*) (Ishihara et al., 2011). We found a significant increase in the concentration of phenolics in *A. violaceofuscus*-associated *H. annuus* and *G. max* as related to non-associated seedlings, thus verified the effort of Abd_Allah et al. (2018) that symbiotic endophytes enhance phenolics content of their host plant under abiotic stresses. *A. japonicas* (Ismail et al., 2018) and *A. flavis* (Ismail et al., 2019) in previous studies have shown almost similar effect on the production

of flavonoids, phenolics and proline in *H. annuus* and *G. max* under heat stress.

Furthermore, AAO and CAT are known to have role as antioxidants against ROS generating during stress. AAO and CAT not only detoxify $H_2O_2$ synthesized in mitochondria and microbodies of plants in stressful conditions but also regulate stress reactions in all plants. We found low amount of AAO and CAT in *A. violaceofuscus*-associated *H. annuus* and *G. max* subjected to heat stress, hence approved the work of Ismail et al. (2018). On the contrary, higher activity of CAT and AAO were observed in the *A. flavis* (Ismail et al., 2019) associated *H. annuus* and *G. max* subjected to heat stress. This shows the ability of *A. violaceofuscus* to control the activity of CAT and AAO in host plant species under heat stress.

## Conclusion

High temperature stress is among the critical restraints to agronomic crops, including *G. max* and *H. annuus* This effort testified a heat-tolerant fungal strain, *A. violaceofuscus* (MH577055) that not only enhanced growth in *H. annuus* and *G. max*, but also facilitated it to resist heat stress by boosting ROS scavenging antioxidant system of host plants, like flavonoids, proline, phenolics, AAO and CAT. *A. violaceofuscus* also the improved the concentration of lipids, proteins and sugars in heat stressed *H. annuus* and *G. max*. Additionally, the production of ABA was lower in the *A. violaceofuscus* associated-*H. annuus* and *G. max* as compared to the *A. japonicas* and *A. flavis* associated plants under heat stress making it the best strain to be as biofertilizer in crops undergoing heat stress.

### Author contributions

Formal analysis: Muhammad Hamayun, Amjad Iqbal, Sumera Afzal Khan; Investigation: Ismail, Anwar Hussain; Supervision: Muhammad Hamayun, Anwar Hussain; Writing the original draft: Ismail, Anwar Hussain; review and editing: Muhammad Hamayun, Amjad Iqbal and In-Jung Lee.

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### Conflict of interest

No potential conflict of interest was reported by the authors.

## References

Ab_Allah, E.F., Alqarawi, A.A., Hashem, A., Radhakrishnan, R., Al-Huqail, A.A., Al-Otibi, F.O.N., Malik, J.A., Alharbi, R.I., Egamberdieva, D., 2018: Endophytic bacterium Bacillus subtilis (BERA 71) improves salt tolerance in chickpea plants by regulating the plant defense mechanisms. J. Plant Interact. 13, 37-44. DOI: 10.1080/17429145.2017.1414321

Ali, S., Khan, S.A., Hamayun, M., Iqbal, A., Khan, A.L., Hussain, A., Shah, M., 2019: Endophytic fungi from Caralluma acutangula can secrete plant growth promoting enzymes. Fresen. Environ. Bull. 28, 2688-2696.

Ashraf, M., Foolad, M., 2007: Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot. 59, 206-216. DOI: 10.1016/j.envexpbot.2005.12.006

Bates, L.S., Waldren, R.P., Teare, I., 1973: Rapid determination of free proline for water-stress studies. Plant Soil 39, 205-207. DOI: 10.1007/BF00018060

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

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. DOI: 10.1007/s13199-018-0545-4

Cai, Y., Luo, Q., Sun, M., Corke, H., 2004: Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 74, 2157-2184. DOI: 10.1016/j.lfs.2003.09.047

El Far, M.M., Taie, H.A., 2009: Antioxidant activities, total anthocyanins, phenolics and flavonoids contents of some sweetpotato genotypes under stress of different concentrations of sucrose and sorbitol. Austral. J. Basic Appl. Sci. 3, 3609-3616.

Hamayun, M., Hussain, A., Khan, S.A., Irshad, M., Khan, A.L., Waqas, M., Shahzad, R., Iqbal, A., Ullah, N., Rehman, G., 2015: Kinetin modulates physio-hormonal attributes and isoflavone contents of Soybean grown under salinity stress. Frontiers Plant Sci. 6, 377. DOI: 10.3389/fpls.2015.00377

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. Frontiers Microbiol. 8, 686. DOI: 10.3389/fmicb.2017.00686

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. DOI: 10.1016/j.chemosphere.2018.07.197

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. DOI: 10.1371/journal.pone.0208150

Iqbal, M., Ashraf, M., 2013: Gibberellic acid mediated induction of salt tolerance in wheat plants: Growth, ionic partitioning, photosynthesis, yield and hormonal homeostasis. Environ. Exp. Bot. 86, 76-85. DOI: 10.1016/j.envexpbot.2010.06.002

Ishihara, A., Nakao, T., Mashimo, Y., Murai, M., Ichimaru, N., Tanaka, C., Nakajima, H., Wakasa, K., Miyagawa, H., 2011: Probing the role of tryptophan-derived secondary metabolism in defense responses against Bipolaris oryzae infection in rice leaves by a suicide substrate of tryptophan decarboxylase. Phytochem. 72, 7-13. DOI: 10.1016/j.phytochem.2010.11.001

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. DOI: 10.1155/2019/1295457

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. DOI: 10.1155/2018/7696831

Kang, S.-M., Hamayun, M., Khan, M.A., Iqbal, A., Lee, I.-J., 2019: *Bacillus subtilis* JW1 enhances plant growth and nutrient uptake of Chinese cabbage through gibberellins secretion. J. Appl. Bot. Food Qual. 92, 172-178. DOI: 10.5073/JABFQ.2019.092.023

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. DOI: 10.1186/1471-2180-8-231

Lorenzo, O., Chico, J.M., Sánchez-Serrano, J.J., Solano, R., 2004: JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell 16, 1938-1950. DOI: 10.1105/tpc.022319

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Luck, H., 1974: Methods in Enzymatic Analysis II (ed.) Bergmeyer.(Publ.). Academic Press, New York.

Mehmood, A., Hussain, A., Irshad, M., Hamayun, M., Iqbal, A., Khan, N., 2019a: In vitro production of IAA by endophytic fungus *Aspergillus awamori* and its growth promoting activities in *Zea mays*. Symbiosis 77, 225-235. DOI: 10.1007/s13199-018-0583-y

Mehmood, A., Hussain, A., Irshad, M., Hamayun, M., Iqbal, A., Rahman, H., Tawab, A., Ahmad, A., Ayaz, S., 2019b: Cinnamic acid as an inhibitor of growth, flavonoids exudation and endophytic fungus colonization in maize root. Plant Physiol. Biochem 135, 61-68. DOI: 10.1016/j.plaphy.2018.11.029

Mhamdi, A., Queval, G., Chaouch, S., Vanderauwera, S., Van Breusegem, F., Noctor, G., 2010: Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. J. Exp. Bot. 61, 4197-4220. DOI: 10.1093/jxb/erq282

Misra, N., Dwivedi, U., 2004: Genotypic difference in salinity tolerance of green gram cultivars. Plant Sci. 166, 1135-1142. DOI: 10.1016/j.plantsci.2003.11.028

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

Muhammad, I., Niaz, A., Gul, J., Amjad, I., Muhammad, H., Farzana, G.J., Anwar, H., In-Jung, L., 2019: *Trichoderma reesei* improved the nutrition status of wheat crop under salt stress. J. Plant Interact. 14, 590-602. DOI: 10.1080/17429145.2019.1684582

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. DOI: 10.1016/j.plaphy.2019.04.019

Oberbacher, M., Vines, H., 1963: Spectrophotometric assay of ascorbic acid oxidase. Nature 197, 1203-1204. DOI: 10.1038/1971203a0

Park, H.L., Lee, S.-W., Jung, K.-H., Hahn, T.-R., Cho, M.-H., 2013: Transcriptomic analysis of UV-treated rice leaves reveals UV-induced phytoalexin biosynthetic pathways and their regulatory networks in rice. Phytochem. 96, 57-71. DOI: 10.1016/j.phytochem.2013.08.012

Quan, L.J., Zhang, B., Shi, W.W., Li, H.Y., 2008: Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. J. Integr. Plant Biol. 50, 2-18. DOI: 10.1111/j.1744-7909.2007.00599.x

Rodriguez, R., White Jr, J., Arnold, A.E., Redman, a.R.a., 2009: Fungal endophytes: diversity and functional roles. New phytologist 182, 314-330. DOI: 10.1111/j.1469-8137.2009.02773.x

Rodriguez, R.J., Woodward, C.J., Redman, R.S., 2012: Fungal influence on plant tolerance to stress. In: Southworth, D. (ed.), Biocomplexity of Plant-Fungal Interactions. DOI: 10.1002/9781118314364.ch7

Su, Y., Guo, J., Ling, H., Chen, S., Wang, S., Xu, L., Allan, A.C., Que, Y., 2014: Isolation of a novel peroxisomal catalase gene from sugarcane, which is responsive to biotic and abiotic stresses. PLoS One 9, e84426. DOI: 10.1371/journal.pone.0084426

Sun, C., Johnson, J.M., Cai, D., Sherameti, I., Oelmüller, R., Lou, B., 2010: Piriformospora indica confers drought tolerance in Chinese cabbage leaves by stimulating antioxidant enzymes, the expression of drought-related genes and the plastid-localized CAS protein. J. Plant Phys. 167, 1009-1017. DOI: 10.1016/j.jplph.2010.02.013

Toh, S., Imamura, A., Watanabe, A., Nakabayashi, K., Okamoto, M., Jikumaru, Y., Hanada, A., Aso, Y., Ishiyama, K., Tamura, N., 2008: High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in Arabidopsis seeds. Plant Phys. 146, 1368-1385. DOI: 10.1104/pp.107.113738

Van Handel, E., 1985: Rapid determination of total lipids in mosquitoes. J. Am. Mosq. Control Assoc. 1, 302-304.

Waqas, M., Khan, A.L., Kamran, M., Hamayun, M., Kang, S.-M., Kim, Y.-H., Lee, I.-J., 2012: Endophytic fungi produce gibberellins and indoleacetic acid and promotes host-plant growth during stress. Molecules 17, 10754-10773. DOI: 10.3390/molecules170910754

Warrier, R., Paul, M., Vineetha, M., 2013: Estimation of salicylic acid in Eucalyptus leaves using spectrophotometric methods. Genetics Plant Physiol. 3, 90-97.

Yoon, J.Y., Hamayun, M., Lee, S.-K., Lee, I.-J., 2009: Methyl jasmonate alleviated salinity stress in soybean. J. Crop Sci. Biotechnol. 12, 63-68. DOI: 10.1007/s12892-009-0060-5

Zahid, K.R., Ali, F., Shah, F., Younas, M., Shah, T., Shahwar, D., Hassan, W., Ahmad, Z., Qi, C., Lu, Y., Iqbal, A., Wu, W., 2016: Response and tolerance mechanism of cotton *Gossypium hirsutum* L. to elevated temperature stress: a review. Front. Plant Sci. 7, 937. DOI: 10.3389/fpls.2016.00937

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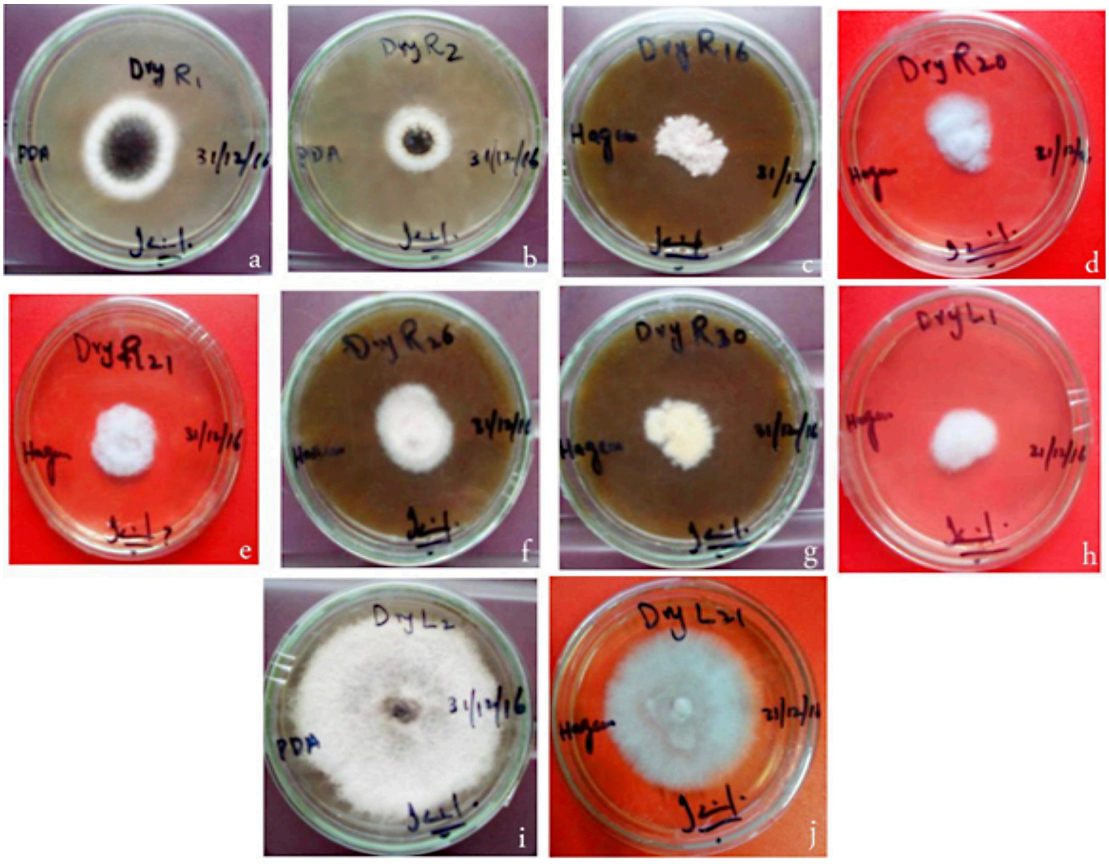


**Fig. S1:** Colonies of endophytic fungi grown on Hagam minimal medium and purified on PDA media plates, isolated from *Dryopteris* L. Ten different fungal colonies were isolated from the host plant, 7 from roots and 3 from leaves i.e. DryR-1, DryR-2, DryR-16, DryR-20, DryR-21, DryR-26, DryR-30, DryL-1, DryL-2 and DryL-21 (DryR represents *Dryopteris* L. root while DryL represents *Dryopteris* L. leaf).

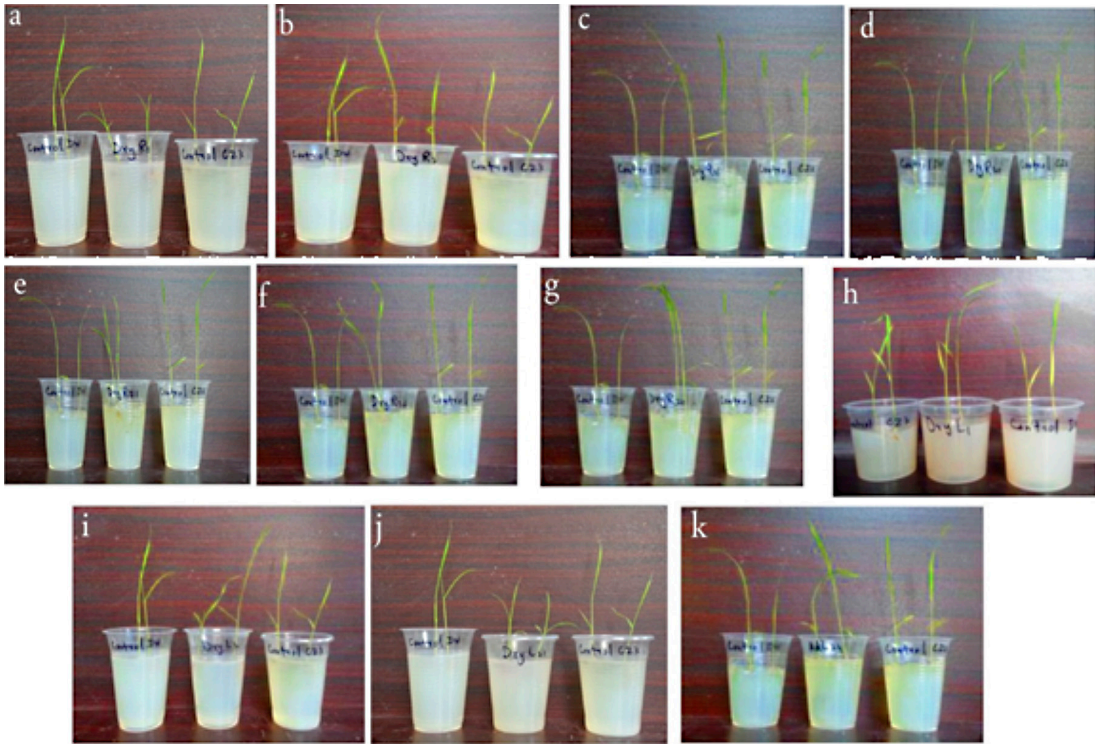


**Fig. S2:** Preliminary screening of fungal filtrates (100 µl) isolated from *Dryopteris* L. on rice seedlings at 2 leaves stage grown in 0.8% water-agar medium for 2 weeks at 25 °C. Reading taken after 1 week of culture filtrate application. Each set has 3 treatments. including Czapek control (right), distilled water control (left) and endophyte cultural filtrate (middle).