**Aspergillus foetidus Regulated the Biochemical Characteristics of Soybean and Sunflower under Heat Stress Condition: Role in Sustainability**

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**Abstract:** Plants are susceptible to various environmental constrains, including heat stress due to their sessile nature. Endophytic fungi can be used as a novel technique to protect crop plants against the injurious effects of thermal stress. Endophytic fungi were isolated from *Adiantum capillus-veneris* L. and tested against heat stress in *Glycine max* L. and *Helianthus annuus* L. The results exhibited increased levels of the plant’s chlorophyll, height and biomass in *Aspergillus foetidus* (AdR-13) inoculated host crop species. Conversely, a significant decrease in lipid peroxidation and reactive oxygen species (ROS) production was noted in *A. foetidus*-associated host crop species. Likewise, the amounts of ROS-degrading antioxidants (glutathione reductase (GR), peroxidase (POD), ascorbic acid oxidase (AAO), superoxide dismutase (SOD) and catalase (CAT)) as well as phenolics were increased, while the amounts of proline and abscisic acid (ABA) were decreased in fungal-associated test crops. Total lipids, proteins and sugars were noted to be high in *A. foetidus*-associated test crops. From the results, we concluded that *A. foetidus* have a role in heat stress mitigation that might help to sustain the production of important crops in the future.

**Keywords:** endophytic fungi; *A. foetidus*; *Adiantum capillus-veneris* L.; heat stress; antioxidants; ROS

1. **Introduction**

In recent times, crop production has been badly affected by various abiotic stresses, including heat stress [1–3]. Such elevation in worldwide temperature may lead to saline as well as drought conditions. The exposure of plants to one or a combination of these stresses can cause stunted growth and low yield [3,4]. In fact, plants can respond to such stresses in terms of high reactive oxygen species (ROS) production [5]. The negative effects of the ROS can be controlled by plant’s native antioxidant system [6]. To evade oxidative injuries, plants have well-defined antioxidant [7] in the form of self-activated-defense-mechanisms (SADMs). Generally, the antioxidant system of the living species can be divided in to enzymatic and non-enzymatic system. Both systems might work in harmony in order to reclaim ROS. Enzymatic antioxidant system encompasses glutathione reductase (GR), ascorbic acid oxidase (AAO), peroxidase (POD), superoxide dismutase (SOD) and catalase...
(CAT), whereas the non-enzymatic system comprises vitamin E, vitamin C, secondary metabolites, etc. [8]. Besides the enzymatic and non-enzymatic antioxidant systems, plant hormones might help the plant species to combat various biotic and abiotic stresses [9–11]. ABA, for example helps the plants to re-regulate the opening and closing of the stomata after sensing heat or drought stresses [12].

Higher plants can accumulate proline and phenolics under stressful conditions. The function of prolines and phenolics are to help in the stabilization of proteins and cellular membranes. They can also act as a ROS scavengers and buffer the cellular redox reactions under stress [13–15]. Additionally, proline necessitates the cellular metabolism, protein compatible hydrotrope and sustenance of cellular-acidosis by complementing the nicotineamide-adenine dinucleotide phosphate (NADP+)/Dihydronicotinamide-adenine dinucleotide phosphate (NADPH)ratio [16]. Once the plant get relieved from stressful condition, proline gets metabolised with the generation of massive amounts of reducing agents employed for adenosine triphosphate (ATP) production during oxidative phosphorylation in mitochondria to repair the damage [16].

Generally, plants are very susceptible to heat stress and, in crop plants, this is devastating to a country’s economy. Sunflower and soybean are important oil crops that are grown almost everywhere in the world for revenue. The optimal temperature for sunflower growth and development is around 25–28 °C [17,18], whereas the optimal temperature for soybean growth and development is 22–25 °C [19]. Both crops are heat sensitive and requires the development of resilient varieties to support the economy of an agriculture based country. However, the release of new resistant cultivars by means of classical breeding is laborious and time consuming, while the advanced molecular techniques are highly expensive. On the contrary, the use of endophytes in crop production under stress conditions is cheaper and less time consuming.

Endophytes can live inside the host tissues in a harmony and do not causes any harm to the host [20]. Being a symbiont the endophytes can restore the belted plant growth under stressful conditions by endowing resistance, minimizing the effect of diseases, hasten the assimilation of vital minerals and recuperate the biomass synthesis of the host plant [21]. Plants without accommodating the important endophytes in their tissues can be vulnerable to the environmental factors that hamper its growth [1,22]. Aspergillus sydowii was formerly known to build up the capacity of the host plant to resist noxious effect of the heavy metals by modulating the behaviour of lipid peroxidation, GR, POD, AAO, SOD, CAT and the contents of proline [2,23]. We investigated the characteristic of the A. foetidus (an endophytic fungus isolated from Adiantum capillus-veneris) in attributing resistance against high temperature stress in crop species. i.e., G. max and H. annuus. As mentioned earlier, both species are among the top edible oil producing crops around the world. Both crops are heat sensitive and facing high temperature stress now-a-day, therefore, we selected it as test crops in the present study.

2. Materials and Methods

The seeds of H. annuus (ICI Hyson 33) and G. Max (Swat 84) was sown in small plastic pots holding 100 g of sterilized sand. The pots were kept in two different growth chambers for two weeks. The temperature of the one growth chamber was maintained at 25 °C and the other at 40 °C. A light intensity of 472.5 μmol m⁻² s⁻¹, 12 h of day/night cycle, 70% relative humidity (RH) was maintained in growth chambers. All the experiments were performed in triplicates.

2.1. Isolation of the Fungal Endophyte AdR-13

A. capillus-veneris was used for the isolation of endophytes according to the standardized protocol of Benizri et al. [24]. A. capillus-veneris is a wild species and can be found in places that undergo hot summer. It is due to heat standing ability that we have selected A. capillus-veneris to isolate endophytes for the amelioration of heat stress. Initially, plant roots of A. capillus-veneris were collected in plastic zip bags and brought to the laboratory.
The roots were carefully washed with ordinary tap water to eliminate dirt. Ethanol (70%) was used first for 2 min in order to sterilize the clean roots, then the roots were treated with sodium hypochlorite (5%) for 5 min. To remove traces of ethanol and NaOCl, the roots were washed with autoclaved distilled water. The sterilized roots were cut into pieces (1–2 cm) and 5 pieces of cut roots per plate were employed in a Hagem medium plate. The plates were shifted to the incubator, where they were incubated at 28 °C till the emergence of the fungal colonies. Individual colonies were obtained by repeated culturing on potato dextrose agar (PDA) plates.

2.2. Early Assessment of the Potent Fungi Using Oryza sativa

The potency of fungal strain was initially checked in O. sativa seedlings. Fresh biomass of the isolated fungal endophyte (AdR13) was added to a flask holding 50 mL of the Czapek broth. The flask was placed in a shaking-incubator at 28 °C for a week, and 120 rpm was maintained till the end of the experiment. After one week of incubation, the Czapek medium was filtered and the culture filtrate was collected for further use. O. sativa variety Fakhr-e-Malakand was taken and the collected culture filtrate (100 µL) was applied to the apex of the seedlings already flourishing in water-agar medium (0.8%). After a week of incubation, the growth attributes of the O. sativa seedlings (i.e., total chlorophyll, fresh and dry weights of root and shoot, and root and shoot length) were calculated. O. sativa seedlings from control treatments were treated with distilled water or Czapek broth [25].

2.3. Identification of AdR13

The method of Khan et al. [26] with minor modification was adopted for the identification of AdR13 endophyte. The primers ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') were utilised in the amplification of 18S rRNA. To find sequence homology approximation, the BLASTn1 program (National Institute of Health (NIH) Rockville Pike, Bethesda MD, USA) was used. The phylogenetic consensus tree was constructed through the neighbor joining (NJ) method using MEGA X software [27].

The method of Khan et al. [26] was adopted for the isolation of AdR13 DNA. Initially, a microfuge was taken and transferred 500 µL of 5% of sodium dodecyl sulfate in bead beating solution. The mycelium (200 mg) of AdR13 was then added to the microfuge tube and the contents of the tube were mixed by using vortex. suspended in the microfuge tube containing 500 µL of a bead beating solution. After mixing the contents of the microfuge tube, it was transferred to the centrifuge, where it was centrifuged at 4 °C and 11,000×g for 10 min. The supernatant (0.5 mL) was collected with great care and transferred to a fresh tube. To the supernatant, we added equal volumes of phenol: chloroform: isooamyl alcohol (25:24:1) and the contents were mixed with the help of the vortex. The vortexed sample was centrifuged again for 5 min at 10,000×g. Isopropanol (2.5 mL) was then added to the collected supernatant and the tube was transferred to the refrigerator, where it was kept for an hour. After incubation in the refrigerator the contents of the tube was centrifuged for 10 min at 14,000×g. The DNA was precipitated in the form of pellets. Cold ethanol (70%) was used to wash the pellet in order to wash out all the impurities. After air drying, 40 µL of Tris-ethylene diamine tetra acetic acid (TE) buffer was added to re-suspend the pellets and the DNA was quantified at 260 nm through Thermo Scientific Nano Drop spectrophotometer [27].

2.4. Inoculation of A. foetidus to G. max and H. annuus

Fresh fungal biomass (1 mg) was mixed with 100 g of autoclaved sand. After thorough mixing of fungal biomass with autoclaved sand, it was transferred to the pots. To each pot, 9 seeds of H. annuus or G. max were sown. The pots were moved to the growth chambers for 14 days. The temperature of the growth chambers was maintained at 25 °C or 40 °C. Hoagland solution (10 mL; Half strength) was employed to the plants at two day intervals until the end of the experiment. The growth attributes were finally measured after the
termination of the experiment [28,29]. Lengths of plants were measured with a scale, whereas fresh weight of plants were measured with the help of an analytical balance. To measure the plant dry weight, the roots and shoot of the test species were initially dried in an oven operated at 105 °C ± 1 for 12 h. The dried weight was then measured using the analytical balance. A chlorophyll meter (Spad-502 plus, Konica Minolta, Tokyo, Japan) was used to compute the chlorophyll contents in the test crop species.

2.5. Estimation of Antioxidants

The protocol of Luck [30] was adopted to estimate the activity of catalase in test crop species, i.e., *H. annuus* and *G. max*. Leaves of the test crop species (2 g) were crushed in a 10 mL of phosphate buffer. The resultant homogenate was collected and centrifuged for 5 min at 10,000 rpm. The supernatant (40 µL) was carefully moved to a tube containing H₂O₂-phosphate buffer (3 mL) with the help of micropipette. After mixing the contents of the tube, absorbance (Abs. = 240 nm) was measured with a spectrophotometer. Estimation of the peroxidase activity (enzyme units/mg protein) was carried out by the method of Kar and Mishra [31]. Approximately, 20× diluted enzyme extract (1 mL) was mixed with 4 mL of 50 µmoles H₂O₂ + 50 µmoles pyrogallol + 125 µmoles of phosphate buffer (pH 6.8). Incubation of the mixture was done at 25 °C for 5 min. H₂SO₄ (5% v/v) was then added to stop the reaction and the concentration of purpurogallin was estimated with the help of spectrophotometer (Abs. = 420 nm). The activity of ascorbate oxidase was measured by a well established protocol [32]. Leaves of the test crop species (0.1 g) was crushed in 2 mL of phosphate buffer and the mixture was centrifuged at 3000 × g for 5 min. The collected supernatant (100 µL) was mixed with a substrate solution (3 mL). The substrate solution comprised ascorbic acid (8.8. mg) and phosphate buffer of pH 5.6 (300 mL). The absorbance (Abs. = 265 nm) was observed for 5 min at intervals of 30 s with the help of the spectrophotometer. The superoxide dismutase activity in the test crops was calculated by the procedure of Beyer Jr and Fridovich [33]. Fresh leaves (0.5 g) of the test crop species were taken in a mortar and crushed with the help of pestle in a 1 mL of potassium phosphate (50 mM) + ethylenediaminetetraacetic acid (EDTA) (1 mM; pH 7.5). The resulting homogenate was transferred to centrifuge tubes for centrifugation. This step was done at 43,000 × g and 4 °C for 15 min. The collected supernatant (50 µL) was then consumed to estimate the activity of SOD by mixing it with a reaction mixture (1 mM EDTA, 13 mM L-methionine, 75 µM nitro blue tetrazolium (NBT) in 50 mM potassium phosphate buffer (pH 7.8)). A 2 µM of riboflavin was then added to initiate the reaction. The SOD activity was measured as an increase in the optical density of nitroblue tetrazolium (NBT) after 1 min of the reaction under standard conditions at 560 nm. The amount of SOD was determined as enzyme units/mg protein. An enzyme unit is defined as the amount of protein consumed to cause 50% inhibition of NBT reduction. The method of Carlberg and Mannervik [34] was adopted to measure the activity of glutathione reductase. The GR activity was characterized by the reduction of glutathione (GSSG) by NADPH at an optical density of 340 nm. Initially, a reaction mixture was made, composed of NADPH (0.125 mmol/L), GSSG (1 mmol/L), EDTA (1 mmol/L) in potassium phosphate buffer (100 mmol/L; pH 7.0). Fresh leaves of the test crops were crushed in the mortar with help of pestle in the presence of liquid nitrogen, EDTA (1 mmol/L), Triton X-100 (0.1% v/v), dithiothreitol (2 mmol/L) in 100 mmol/L Tris-HCl (pH 8). The homogenate was centrifuged at 27,000 × g to collect the supernatant. Added, 200 µL of the sample supernatant to 0.8 mL of the reaction mixture and the optical density was observed with the help of spectrophotometer (Abs. = 340 nm).

2.6. Estimation of ABA

Estimation of ABA in test crop species was undertaken according to the method of Yoon et al. [12]. Fresh leaves (0.5 g) of *H. annuus* and *G. max* were pulverized in liquid N₂. The homogenate was then added to the 2 mL mixture, composed of isopropanol (1.5 mL) and glacial acetic acid (28.5 mL). The resultant mixture was concentrated in a
rotary evaporator and then filtered. Diazomethane was added to the mixture and was 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 Quest, 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.

2.7. Estimation of Phenolics and Proline

Estimated total phenolics in test crop species were measured by the established protocol of Cai et al. [35]. To the sample (0.2 mL), 0.5 N of the Folin–Ciocalteu reagent was added and the mixture was kept for 4 min at 25 °C. Sodium carbonate (75 g/L) was then added and the contents were heated for 1 min at 100 °C. The heated mixture was transferred to the dark place and incubated for 2 h. After 2 h of incubation, the absorbance was measured at 650 nm. Gallic acid (Sigma Aldrich, Peshawar, Pakistan) was used as a standard. The concentration of proline in the test crops were estimated by an established method [36], but with minor modifications. Fresh leaves (0.1 g) of the test crops were pulverized in 3% of sulfo-salicylic acid (4 mL). The pulverized tissues in sulfo-salicylic acid was incubated for 24 h at 5 °C. After incubation, the homogenate was centrifuged at 3000 rpm for 5 min. A supernatant (2 mL) was mixed with acid ninhydrin (2 mL) and heated at 100 °C for 1 h. Toluene (4 mL) was finally added to the mixture and the absorbance was checked with the help of spectrophotometer (Abs. = 520 nm). Pure proline (Sigma Aldrich) was used as standard.

2.8. Estimation of Total Lipids, Proteins and Sugars

The protocol of Lowry et al. [37] was adopted to estimate the total proteins in the test crops. The filtered leaf homogenate of the test crops (0.1 mL) was added to 0.1 mL of NaOH (2 M) and then hydrolyzed at 100 °C. The hydrolysate was allowed to cool and then added to 1 mL of complex-forming solution (complex-forming solution = 1% (w/v) copper sulphate in distilled water, 2% (w/v) sodium carbonate in distilled water, 2% (w/v) sodium potassium tartrate in distilled water). The resultant mixture was allowed to stand for 10 min at room temperature. Folin reagent (1 mL) was added to the mixture and the solution was vortexed. The vortexed sample was again allowed to stand for 30 min at room temperature and the absorbance was observed with a spectrophotometer (Abs. = 650 nm). Bovine serum albumin (Sigma Aldrich) was used as a standard. The methodology of Van Handel [38] was adopted to estimate the total lipids in H. annuus and G. max seedlings. Leaves of the test crop species were crushed in 1 mL of 2% sodium sulfate. The homogenate was then centrifuged for 5 min at 11,000 × g. About 1 mL of the supernatant was transferred to the tubes containing 1 mL chloroform/methanol (1:2 ratio v/v). The mixture was evaporated at 90 °C on a water bath until precipitates formed. The precipitates were then dissolved by using 2 mL of concentrated sulfuric acid. The mixture was heated for the second time at 90 °C for 20 min on a water bath. After cooling, 5 mL of vanillin-phosphoric acid was added and the mixture was left on a bench for half and hour for color development. The absorbance was ultimately recorded using spectrophotometer (Abs. = 525 nm). Pure canola oil was used as a standard. The well-established method of Mohammadkhani and Heidari [39] was adopted to estimate the soluble sugars in the leaves of test crops. Leaves (0.5 g) were crushed in a mortar and pestle in the presence of liquid nitrogen. The resultant homogenate was mixed with distilled water (5 mL) and the mixture was centrifuged for 5 min at 4000 × g. Supernatant (0.1 mL) was collected in fresh tube and we added 1 mL of 80% phenol. The mixture was allowed to stand for 10 min at room temperature. After incubation, 5 mL of concentrated H2SO4 was added to the mixture and was then incubated at room temperature for an hour. The absorbance was finally observed at 485 nm. Glucose (Sigma Aldrich) was used as a standard.
2.9. Statistical Analysis

The data were analyzed by the analysis of variance. The significantly different means were separated by the Duncan multiple range test at \( p = 0.05 \), using SPSS-20 (SPSS Inc., Chicago, IL, USA). Moreover, the significant means were denoted by different letters as a superscript.

3. Results

3.1. Isolation and Plant Growth Promoting Activity of Endophytes

From the roots of *A. capillus-veneris*, potent endophytic fungi were isolated that were coded as AdR-13 before identification. The potency of the isolated stain was initially screened for growth promoting activity in *O. sativa* seedlings. The *O. sativa* seedlings inoculated with the endophytic strain (AdR-13) had longer roots and shoots as compared to the non-inoculated controls (Table 1). Similarly, the fresh and dry weight of the *O. sativa* seedlings inoculated with the endophytic strain (AdR-13) were higher when compared with the control seedlings. Higher chlorophyll contents were also noticed in the AdR-13 associated *O. sativa* seedlings (Table 1). After observing a positive impact of the isolated endophyte (AdR-13) on *O. sativa*, the strain was further utilized to check its growth-promoting activity in *G. max* and *H. annuus* exposed to 25 °C and 40 °C. The results revealed that the isolated AdR-13 strain has little effect on the chlorophyll contents, root and shoot lengths and weights of *G. max* exposed to 25 °C and 40 °C (Table 2). Similar observations were recorded in AdR-13 associated *H. annuus* exposed to 25 °C and 40 °C (Table 3).

### Table 1. Effect of *A. foetidus* filtrate on the growth of *O. sativa* seedlings.

| Growth Attributes       | Ctrl (DW)       | Ctrl (Czk)      | *A. foetidus* |
|-------------------------|-----------------|-----------------|--------------|
| SL (cm)                 | 10.4 ± 0.8 \(^a\) | 10.6 ± 1.5 \(^a\) | 15 ± 0.6 \(^b\) |
| RL (cm)                 | 4.9 ± 0.8 \(^a\)  | 6.3 ± 0.3 \(^a\)  | 8 ± 0.6 \(^b\)  |
| SFW (g)                 | 0.03 ± 0.0002 \(^a\) | 0.0317 ± 0.0003 \(^a\) | 0.0409 ± 0.0002 \(^b\) |
| RFW (g)                 | 0.08 ± 0.007 \(^a\) | 0.082 ± 0.006 \(^a\) | 0.1031 ± 0.005 \(^b\) |
| SDW (g)                 | 0.0047 ± 0.0003 \(^a\) | 0.0043 ± 0.0003 \(^a\) | 0.0063 ± 0.0009 \(^b\) |
| RDW (g)                 | 0.0137 ± 0.0009 \(^a\) | 0.015 ± 0.0001 \(^a\) | 0.020 ± 0.0006 \(^b\) |
| Chlorophyll content (SPAD) | 18.9 ± 1.3 \(^a\) | 21.4 ± 0.3 \(^a\) | 23.4 ± 0.7 \(^b\) |

SL = shoot length; RL = root length; SFW = fresh weight of shoots; RFW = fresh weight of roots; SDW = dry weight of shoots; RDW = dry weight of roots; Ctrl = control; Czk = Czapek medium, DW = distilled water. Data are mean of 3 replicates with standard error. Data that is followed by different letter (i.e., \(^a,b\)) is significantly different (\( p = 0.05 \)) as estimated by Duncan’s multiple range test.

### Table 2. Effect of *A. foetidus* on the growth features of *G. max*.

| Growth Attributes | 25 °C | 40 °C | *A. foetidus* | Control | *A. foetidus* |
|-------------------|-------|-------|---------------|---------|---------------|
| SL (cm)           | 39 ± 1.7 \(^b\) | 40 ± 0.6 \(^b\) | 26 ± 0.9 \(^a\) | 27 ± 2.0 \(^a\) |
| RL (cm)           | 13 ± 1.4 \(^a,b\) | 14 ± 1.4 \(^b\) | 10 ± 0.6 \(^a\) | 11 ± 0.3 \(^a,b\) |
| SFW (g)           | 1.12 ± 0.02 \(^a\) | 1.47 ± 0.20 \(^a\) | 0.82 ± 0.04 \(^a\) | 1.19 ± 0.06 \(^a\) |
| RFW (g)           | 0.18 ± 0.02 \(^a\) | 0.25 ± 0.09 \(^a\) | 0.13 ± 0.02 \(^a\) | 0.16 ± 0.02 \(^a\) |
| SDW(g)            | 0.14 ± 0.012 \(^b\) | 0.14 ± 0.0051 \(^b\) | 0.08 ± 0.001 \(^a\) | 0.11 ± 0.009 \(^a\) |
| RDW (g)           | 0.075 ± 0.002 \(^b\) | 0.078 ± 0.003 \(^b\) | 0.01 ± 0.001 \(^a\) | 0.012 ± 0.001 \(^a\) |
| Chlorophyll (SPAD) | 31 ± 0.2 \(^a\) | 33 ± 1.6 \(^a\) | 29 ± 2.3 \(^a\) | 32 ± 3.1 \(^a\) |

Effect of *A. foetidus* on *G. max* seedlings, isolated from *A. capillus-veneris*. SL = shoot length; RL = root length; SFW = fresh weight of shoots; RFW = fresh weight of roots; SDW = dry weight of shoots; RDW = dry weight of roots; Ctrl = control. Data are mean of 3 replicates with standard error. Data that is followed by different letter (i.e., \(^a,b\)) is significantly different (\( p = 0.05 \)) as estimated by Duncan’s multiple range test.
### Table 3. Effect of *A. foetidus* on the growth features of *H. annuus*.

| Growth Attributes/ Temperature Stress | 25 °C | 40 °C |
|--------------------------------------|-------|-------|
|                                      | Ctrl  | *A. foetidus* | Ctrl  | *A. foetidus* |
| SL (cm)                              | 23.7 ± 0.9 b,c | 25.9 ± 0.9 c | 20.5 ± 0.4 a | 22.5 ± 0.5 ab |
| RL (cm)                              | 9.3 ± 0.6 b | 10.9 ± 0.5 b | 6.2 ± 0.6 a | 6.7 ± 0.9 a |
| SFW (g)                              | 1.22 ± 0.09 a | 1.32 ± 0.14 a | 0.82 ± 0.31 a | 0.93 ± 0.22 a |
| RFW (g)                              | 0.13 ± 0.021 b,c | 0.14 ± 0.084 c | 0.08 ± 0.074 a | 0.09 ± 0.006 a,b |
| SDW (g)                              | 0.08 ± 0.024 a,b | 0.09 ± 0.001 b | 0.04 ± 0.0001 a | 0.06 ± 0.009 a,b |
| RDW (g)                              | 0.024 ± 0.001 b | 0.032 ± 0.001 c | 0.014 ± 0.001 a | 0.016 ± 0.002 a |
| Chlorophyll (SPAD)                   | 40 ± 4.7 a | 45.6 ± 2.6 a | 38.4 ± 1.9 a | 39 ± 1.3 a |

Effect of *A. foetidus* on *H. annuus* seedlings, isolated from *A. capillus-veneris*. SL = shoot length; RL = root length; SFW = fresh weight of shoots; RFW = fresh weight of roots; SDW = dry weight of shoots; RDW = dry weight of roots; ctrl = control. Data are mean of 3 replicates with standard error. Data that is followed by different letter (i.e., a,b,c) is significantly different (*p* = 0.05) as estimated by Duncan’s multiple range test.

#### 3.2. Molecular Identification of Isolate AdR-13

The BLAST search program (Basic Local Alignment Search Tool, 2012) was used to align the nucleotides of AdR-13 isolate from the the Internal transcribed spacer (ITS) region for comparison. Maximum resemblance with *A. foetidus* was noticed by using 18 S rRNA sequence. From 21 taxa (20 reference and 1 clone), the phylogenetic consensus tree was constructed through the neighbor joining (NJ) method using MEGA X software (Figure 1). The isolated endophyte AdR-13 from *A. capillus-veneris* formed a clad with *A. foetidus* strengthened by 91% bootstrap value in the harmony tree. Sequence homology and phylogenetic analysis verified that *A. foetidus* was our isolate.

![Figure 1](image-url). Identification of fungal isolate AdR-13 with 91% bootstrap value as *Aspergillus foetidus* using the neighbor joining (NJ) method.

#### 3.3. Arbitration of Enzymatic and Non-Enzymatic Antioxidants

The results regarding the enzymatic activity of various enzymes in *G. max* exposed to 25 °C and 40 °C are given in Table 4. The activity of AAO was noted higher in the *A. foetidus* associated *G. max* exposed to 40 °C temperature as compared to the other treatments. Similarly, the activity of other enzymes, i.e., CAT, POD, SOD and GR was recorded high in *A. foetidus* associated *G. max* exposed to 40 °C temperature (Table 4). By contrast, a
non-significant difference ($p = 0.05$) in AAO activity was noticed in *A. foetidus* associated *H. annuus* compared to its respective control at 40 °C (Table 5). However, the activity of the CAT, POD, SOD and GR was significantly ($p = 0.05$) higher in *A. foetidus* associated *H. annuus* as compared to the *A. foetidus* free *H. annuus* exposed to 40 °C temperature (Table 5).

Table 4. Effect of *A. foetidus* on the activity of antioxidant enzymes in *G. max*.

| Growth Attributes | 25 °C | 40 °C |
|-------------------|-------|-------|
|                   | Ctrl  | *A. foetidus* | Ctrl  | *A. foetidus* |
| AAO (EU/mg ptn)   | 1.51 ± 0.09 $^a$ | 1.61 ± 0.07 $^b$ | 1.85 ± 0.12 $^c$ | 2.04 ± 0.14 $^d$ |
| CAT (EU/mg ptn)   | 0.46 ± 0.02 $^a$ | 0.58 ± 0.04 $^b$ | 0.95 ± 0.41 $^c$ | 1.04 ± 0.17 $^d$ |
| POD (EU/mg ptn)   | 1.42 ± 0.03 $^a$ | 1.64 ± 0.11 $^a$ | 3.90 ± 0.22 $^b$ | 5.01 ± 0.64 $^c$ |
| SOD (EU/mg ptn)   | 18 ± 0.64 $^a$ | 19 ± 0.55 $^a$ | 37 ± 0.73 $^b$ | 43 ± 1.34 $^c$ |
| GR (EU/mg ptn)    | 1.54 ± 0.61 $^a$ | 0.16 ± 0.13 $^a$ | 1.86 ± 0.05 $^b$ | 2.09 ± 0.11 $^c$ |

Effect of *A. foetidus* on *G. max* seedlings, isolated from *A. capillus-veneris*. AAO = ascorbic acid oxidase; CAT = catalase; POD = peroxidase; SOD = superoxide dismutase; GR = glutathione reductase; EU = enzyme unit; ptn = protein; Ctrl = control. Data are mean of 3 replicates with standard error. Data that is followed by different letter (i.e., $a,b,c,d$) is significantly different ($p = 0.05$) as estimated by Duncan’s multiple range test.

Table 5. Effect of *A. foetidus* on the activity of antioxidant enzymes in *H. annuus*.

| Growth Attributes | 25 °C | 40 °C |
|-------------------|-------|-------|
|                   | Ctrl  | *A. foetidus* | Ctrl  | *A. foetidus* |
| AAO (EU/mg ptn)   | 0.65 ± 0.06 $^a$ | 0.77 ± 0.05 $^b$ | 2.28 ± 0.36 $^c$ | 2.55 ± 0.28 $^c$ |
| CAT (EU/mg ptn)   | 0.26 ± 0.03 $^a$ | 0.34 ± 0.03 $^b$ | 0.60 ± 0.43 $^c$ | 0.72 ± 0.04 $^d$ |
| POD (EU/mg ptn)   | 1.11 ± 0.08 $^a$ | 1.37 ± 0.26 $^a$ | 2.73 ± 0.16 $^b$ | 3.78 ± 0.18 $^c$ |
| SOD (EU/mg ptn)   | 12 ± 0.47 $^a$ | 13 ± 0.64 $^a$ | 22 ± 1.05 $^b$ | 26 ± 0.78 $^c$ |
| GR (EU/mg ptn)    | 0.89 ± 0.05 $^a$ | 0.94 ± 0.04 $^a$ | 2.11 ± 0.19 $^b$ | 2.63 ± 0.13 $^c$ |

Effect of *A. foetidus* on *H. annuus* seedlings, isolated from *A. capillus-veneris*. AAO = ascorbic acid oxidase; CAT = catalase; POD = peroxidase; SOD = superoxide dismutase; GR = glutathione reductase; EU = enzyme unit; ptn = protein; Ctrl = control. Data are mean of 3 replicates with standard error. Data that is followed by different letter (i.e., $a,b,c,d$) is significantly different ($p = 0.05$) as estimated by Duncan’s multiple range test.

Figure 2 represents the results of flavonoids, phenolics and proline contents of the *G. max* and *H. annuus* (with or without *A. foetidus* association) exposed to 25 °C and 40 °C. A significant ($p = 0.05$) increase in the flavonoids content was noticed in the *A. foetidus* associated *G. max* under 25 °C and 40 °C as compared to the controls (Figure 2A). The increase in flavonoid contents in *A. foetidus* associated *G. max* under 25 °C was 7.5%, whereas a 41% increase in flavonoid contents was noted in *A. foetidus* associated *G. max* under 40 °C as compared to their respective controls (Figure 2A). The value of flavonoids under 25 °C and 40 °C (with or without association) exposed to 25 °C and 40 °C, respectively, as compared to their controls (Figure 2C). An almost double amount (91%) of phenolics was observed in *A. foetidus* associated *H. annuus* at 25 °C, while a 31% increase in phenolics was recorded in *A. foetidus*-associated *H. annuus* at 40 °C (Figure 2D). Conversely, a sizable decrease were noticed in the proline contents of *A. foetidus* associated *G. max* and *H. annuus* exposed to 25 °C and 40 °C. A 13% and 40% decrease in proline contents were recorded in *A. foetidus*-associated *G. max* at 25 °C and 40 °C, respectively, as compared to their respective controls (Figure 2E). Likewise, a decrease of 26% and 25% were noted in the *A. foetidus*-associated *H. annuus* as compared to their respective controls at 25 °C and 40 °C (Figure 2F).
Figure 2. (A) Flavonoid content in G. max, (B) flavonoid content in H. annuus, (C) phenolics content in G. max, (D) phenolics content in H. annuus, (E) proline content in G. max and (F) proline content in H. annuus inoculated with and without A. foetidus. Data are the mean of 3 replicates with standard error. Bars that are represented with different letters (i.e., a–d) are significantly different ($p = 0.05$) as estimated by Duncan’s multiple range test.

Figure 3 presents the results of ABA, $H_2O_2$ and malondialdehyde (MDA) contents of the G. max and H. annuus (with or without A. foetidus association) exposed to 25 °C and 40 °C. A decrease in the amount of ABA (4% and 30%) was noticed in A. foetidus-associated G. max at 25 °C and 40 °C as compared to their respective controls; however, the decrease at 25 °C was non-significant ($p = 0.05$) (Figure 3A). Comparably, a 1% increase in ABA contents have been observed in A. foetidus-associated H. annuus at 25 °C, while a significant ($p = 0.05$) increase in ABA content (25%) was recorded in A. foetidus-associated H. annuus at 40 °C (Figure 3B). Additionally, lower amounts of $H_2O_2$ (14%) were recorded in A. foetidus-associated G. max under 25 °C, whereas a 37% decrease was recorded in A. foetidus-associated G. max under 40 °C (Figure 3C). A. foetidus-associated H. annuus at 25 °C showed 18% decrease in $H_2O_2$ contents, while 42% decrease were recorded in A. foetidus-associated H. annuus at 40 °C (Figure 3D). A 41% and 36% decrease in MDA contents were recorded in A. foetidus-associated G. max as compared to their respective controls at 25 °C and 40 °C, respectively (Figure 3E). Likewise, a decrease of 29% and 37%
in MDA contents were noted in the _A. foetidus_-associated _H. annuus_ as compared to their respective controls at 25 °C and 40 °C (Figure 3F).

Figure 3. (A) ABA content in _G. max_, (B) ABA content in _H. annuus_, (C) _H_2_O_2_ content in _G. max_, (D) _H_2_O_2_ content in _H. annuus_, (E) MDA content in _G. max_ and (F) MDA content in _H. annuus_ inoculated with and without _A. foetidus_. Data are the mean of 3 replicates with standard error. Bars that are represented with different letters (i.e., a–d) are significantly different (_p_ = 0.05) as estimated by Duncan’s multiple range test.

Figure 4 represents the results of total proteins, sugars and lipids of the _G. max_ and _H. annuus_ (with or without _A. foetidus_ association) exposed to 25 °C and 40 °C. An increase in the total protein (2% and 21%) was noticed in _A. foetidus_-associated _G. max_ at 25 °C and 40 °C as compared to their respective controls; however, the increase at 25 °C was non-significant (_p_ = 0.05) (Figure 4A). Similarly, a 6% increase in total proteins was observed in _A. foetidus_-associated _H. annuus_ at 25 °C, while a significant (_p_ = 0.05) increase in total proteins (33%) was recorded in _A. foetidus_-associated _H. annuus_ at 40 °C (Figure 4B). Likewise, higher amounts of sugars (10%) were recorded in _A. foetidus_-associated _G. max_ under 25 °C, whereas 25% increase were recorded in _A. foetidus_-associated _G. max_ under 40 °C (Figure 4C). _A. foetidus_-associated _H. annuus_ at 25 °C showed a 17% increase in total sugars, while the 26% increase in sugar content was recorded in _A. foetidus_-associated _H. annuus_ at 40 °C (Figure 4D). An 11% and 21% increase in total lipids was recorded in
A. foetidus-associated G. max as compared to their respective controls at 25 °C and 40 °C, respectively (Figure 4E). Likewise, an increase of 15% and 19% in total lipids was noted in the A. foetidus-associated H. annuus as compared to the respective controls at 25 °C and 40 °C (Figure 4F).

Figure 4. (A) Protein (ptn) content in G. max, (B) protein (ptn) content in H. annuus, (C) sugars content in G. max, (D) sugars content in H. annuus, (E) lipids content in G. max and (F) lipids content in H. annuus inoculated with A. foetidus. Data are the mean of 3 replicates with standard error. Bars that are represented with different letters (i.e., a–d) are significantly different ($p = 0.05$) as estimated by Duncan’s multiple range test.

4. Discussion

Plant species when they undergo heat stress may defend themselves through biosynthetic responses, such as regulation of membrane lipids, re-adjustment of opening and closing of stomata, activation of antioxidant systems, and production of heat-shock-proteins [40]. Moreover, plants can establish a symbiotic relationship with endophytes, which can help the host plants under stress [21,41]. Endophytes can help the host plants under stress conditions by releasing higher amounts of alkaloids, flavonoids and phenolics [42,43]. By doing this, endophytes can improve the growth attributes and impart resistance to host plants against stress [13,44]. In this study, A. foetidus-associated test crops showed higher resistance against thermal stress than control seedlings. Culture
filtrates of *A. foetidus* were initially assessed on *O. sativa* because of their prompt response to growth-promoting phytohormones, produced by endophytes [43]. Culture filtrates of *A. foetidus* profoundly encouraged the rice growth (length and weight of shoot and roots of host plant species) similar to the *Gliocladium cibotii* [11] and *Penicillium glabrum* [45]. Moreover, *A. foetidus* improved the plant growth parameters of *G. max* and *H. annuus* under thermal stress. *A. foetidus* inoculated *G. max* and *H. annuus* gained higher shoot and roots weight, longer roots and shoots, and higher chlorophyll contents as compared to the non-inoculated control plants. Comparable results have been recorded in previous reports, in which *Gliocladium cibotii* [11] and *Penicillium glabrum* [45] has supported the growth of *G. max* and *H. annuus* after exposure to 40 °C. *A. foetidus* association might accelerate the photosynthetic rate in *G. max* and *H. annuus*, which in turn helped in the promotion of growth attributes of the host plants [1,2]. Our results also confirmed the finding of of Sun et al. [46], who observed resistance in chlorophyll breakdown and sustenance in photosynthetic rate in *Piriformospora indica*-associated host plants under drought stress.

Plant can produce higher levels of phenolics against biotic and abiotic stresses to defend themselves [2]. The buildup of higher amounts of phenolics in *A. foetidus*-inoculated host plant species under 40 °C enabled the host to reduce the stocking of ROS, thus lowering the stress. A similar observation was made by Abd_Hallah et al. [6], who reported that endophytes can help the host plants to flourish under stress. Proline is an organic osmolyte that accumulates in plant species during stressful conditions, like heat or drought stress [47,48]. In our study, elevated amounts of proline were noticed in *A. foetidus*-inoculated *G. max* and *H. annuus* that might have a positive effect on buffering of cellular redox potential, enzymatic system, membrane stability and scavenging of free radicals during stress. In fact, proline can contribute towards the cytoplasmic acidosis and maintenance of NADP+/NADPH ratios [49]. Also, proline helps in delivering the stress-reducing agents in order to repair the stress-provoked damages and to produce ATP [50].

Our results demonstrated the role of endophytic fungi *A. foetidus* in associated *G. max* and *H. annuus* seedlings exposed to 40 °C. This role of *A. foetidus* in associated *G. max* and *H. annuus* seedlings might be linked with nutrients uptake and controlled antioxidant system and ROS declination [51]. During the stress, plant can protect themselves from the threatening effects of free radicals by regulating the activity of antioxidant enzymes (AAO, SOD, POD, GR, CAT) [6]. SOD is one of the vital antioxidant enzymes that can inhibit the Haber–Weiss reaction by producing hydroxyl radicals, thus scavenge superoxide radicals and H₂O₂. Extraordinary levels of POD might have a task of diminishing the lethal effect of stress through lignins biosynthesis [52]. CAT activity has a proven role in boosting the immunity of the plant species against the abiotic stress [53], which might be due to elimination of H₂O₂ [54]. AAO and GR have a vital role in the ascorbate–glutathione cycle, in which both of the enzymes can scavenge the ROS species [55]. In the current study, AAO and GR might have reduced the generation of superoxide radicals in order to protect the photosynthetic-electron transport chain under heat stress [55].

Abscisic acid is a well-known phytohormone that can be produced in plant species during stress. *O. sativa* was the first species in which higher productions of ABA was noticed under thermal stress [56]. The genes that are responsible for the production of ABA are upregulated, whereas the genes that are responsible for the breakdown of ABA are downregulated upon the exposure of plants to heat stress [57]. The results of the present study revealed lower amounts of ABA in *A. foetidus*-associated host plant species (*G. max* and *H. annuus*) exposed to 40 °C as compared to *A. foetidus* free plants. The presence of ABA in lower amounts in *A. foetidus* crop species (*G. max* and *H. annuus*) can be attributed to the ABA synthesis genes that were downregulated or the ABA digesting genes that were upregulated. It is also possible that lower amounts of ABA might result in *A. foetidus*-associated plants due to the involvement of GAs [2,23,29]. Furthermore, higher amounts of plants MDA under stressful conditions points towards the lipid peroxidation. In this study, the *A. foetidus*-associated *G. max* and *H. annuus* had lower levels of MDA as compared to
the non-inoculated plants exposed to 40 °C temperature. The accumulation of abnormal amounts of MDA can trigger membrane desolation, whereas plant species associated with endophytes can keep the MDA levels under check [45]. Previously, low levels of MDA (27.5%) have been noticed in chickpea inoculated with *Bacillus subtilis* BERA 71 and exposed to salt stress [6]. We also found that *A. foetidus* can ease the effects of heat stress by dropping the concentration of H$_2$O$_2$ and MDA. Moreover, heat stress has lifted the hydrogen peroxide levels in plants that ultimately imitate membrane structural integrity and guided the fast lipids peroxidation in non-inoculated host plant species [1]. Higher amounts of total lipids, proteins and sugars were noticed in *A. foetidus*-associated *G. max* and *H. annuus* exposed to 40 °C temperature. These findings reflect the positive role of *A. foetidus*, reinforcing the case for its use as a bio-fertilizer. This type of stress is among the crucial constraints in the production of agricultural crops such as *G. max* and *H. annuus*.

### 5. Conclusions

*A. foetidus* enhanced the growth parameters of *G. max* and *H. annuus* exposed to thermal stress. Also, *A. foetidus* helped the host plants to scavenge ROS and elevation of AAO, POD, CAT, GR and SOD activity. From the results we concluded that *A. foetidus* can be one of the best choices for use as a bio-fertilizer or as a thermal stress-relieving bio-agent in future crop production.

### Author Contributions

I., A.A., S.G., S.A.K. and H.-Y.K. designed and performed all the experiments. A.I. and A.H. analyzed the data and wrote the manuscript. M.H. and I.-J.L. supervised the research. A.I., M.H. and I.-J.L. edited the manuscript and arranged the resources for the work. All authors have read and agreed to the published version of the manuscript.

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Not applicable.

### Informed Consent Statement

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### Data Availability Statement

All the data are included in the manuscript.

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### Conflicts of Interest

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

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