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Original Research

An Endophytic Fungus *Gliocladium cibotii* Regulates Metabolic and Antioxidant System of *Glycine max* and *Helianthus annuus* under Heat Stress

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Abstract

In the present study, we have isolated endophytic fungi *Gliocladium cibotii* from *Verbena officinalis* L. and tested against *Oryza sativa* L. seedlings for growth promotion. The strain was used to help *Glycine max* L. and *Helianthus annuus* L. under thermal stress. Association of *G. cibotii* with *G. max* and *H. annuus* enhanced chlorophyll contents, total biomass and plant height. Also, *G. cibotii* significantly curtailed lipids peroxidation and ROS (reactive oxygen species) in host species exposed to heat stress (40ºC). Furthermore, the concentration of ROS-degrading enzymes, like ascorbic acid oxidase (AAO), catalase (CAT), glutathione reductase (GR), peroxidase (POD) and superoxide dismutase (SOD) were boosted in test crops, while concentration of proline and ABA were inhibited. The phenolics and nutritional value (total lipids, proteins and sugars) of experimental plants were also enhanced. The results conclude that *G. cibotii* can be used as a heat stress mitigating weapon for food crops in the future.

Keywords: antioxidants, endophytic fungi, *G. cibotii,* heat stress, ROS

Introduction

High temperature is an imperative abiotic stress in the present age causing huge loss to agricultural crops. Global warming cause fast water losses from the soil surface that increases the risk of drought and salinity [1]. Different ecological stresses cause over-production of ROS that leads to oxidative damage of chlorophylls, lipids and proteins, and distracts cell functional-integrity. ROS consists of hydroxyl radical (OH), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2)

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and superoxide (O_2^{-1}) [2]. Plants have self-activated-defense-mechanisms (SADMs), including compatible osmolytes and antioxidant systems that protect plant from oxidative damages caused by ROS [3]. Another mechanism to avoid oxidative damage is the transportation and storage of toxic ions to less sensitive tissues [4]. The antioxidant system of the plants that detoxify ROS species is demonstrated by catalase (CAT), glutathione reductase (GR), peroxidase (POD) and superoxide dismutase (SOD), tocopherol, ascorbic acid and some secondary metabolites [5-10].

It is well documented that various abiotic and biotic stresses are neutralized by phytohormones, like abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) [9, 10]. ABA is known to be the chief hormone that actively handle the drought and thermal shocks by regulating the stomatal opening and closing [11, 12]. Stomata remain open at high atmospheric humidity, low concentration of CO_2 and light, whereas dry air, high concentration of CO_2, darkness and stress-induced ABA result in closing of stomata [13]. Seed germination, maturation and senescence of vascular plants, including *Arabidopsis thaliana* L., have been reported to be regulated by ABA [14].

High contents of proline and phenolics are hoarded in higher plants during abiotic stresses and functions as proteins and membranes stabilizer, buffer of cellular redox reactions and as ROS foragers [15-18]. Moreover, proline also help in balancing the ratio of $NADP^+$/ NADPH, which is necessary for cellular metabolism, maintenance of cellular-acidosis and protein-compatible-hydrotrope. Proline has role in stress recovery to revamp the damages caused by various stresses [19].

More than 80% of the plants carrying endophytic fungi as endorsements. The endophytes live inside the plant tissues, either permanently or for the short time with no visible sign of harm. Fungal endophytes have role in restoration of plant growth under various abiotic and biotic shocks by accelerating minerals absorption, decrease illness severity and enhances biomass synthesis of their residing host [20-22]. Without endophytes, plants are more susceptible to biotic and abiotic stresses, including low and high temperatures, drought and salinity [2, 16, 23]. The present work is an attempt to introduce *G. cibotii* as a heat-stress adaptive tool in *Helianthus annuus* L. and *Glycine max* L. Also, the study emphasizes on the possible role of *G. cibotii* in future sustainable agriculture.

Materials and Methods

Plant Collection and Isolation of Endophytic Fungi

A wild plant, *Verbena officinalis* L. was collected from District Nowshera Khyber Pakhtunkhwa, Pakistan for the isolation of fungal endophytes [24]. Hagem media was used for preliminary isolation of fungal endophytes. The isolates were then purified on PDA (potato dextrose agar) media plates. The purified fungal isolates were finally grown in Czapek liquid media ($C_6H_{12}O_6$ 10 gm/L, peptone 10 gm/L, KCl 0.5 gm/L, $MgSO4.7H_2O$ 0.5 gm/L, $FeSO_4.7H_2O$ 0.5 gm/L, pH 7.3±0.2) for the secretion of secondary metabolites and biomass collection, for 7 days in a shaking incubator set at 120 rpm.

Fungal Filtrate Screening on *Oryza sativa* L. Seedlings

Filtrates (100 µl) of endophytic fungi were applied to the top of *O. sativa* L. (a commercial rice variety, Fakhr-e Malakand, provided by the Agriculture Research Station, Mingora, Swat, Pakistan). The plants were cultivated in 0.8% (v/w) water-agar media in a growth-chamber for one week (day; 14 hrs 28ºC±0.3; night; 10 hrs 25ºC±0.3 and 70% relative humidity) and then tested for growth promoting or hindering metabolites at the two leaf stage. Total chlorophyll contents, root-shoot lengths, and fresh and dry weight of fungal filtrate treated seedlings (experimental) were compared with distilled water (DW) and Czapek (Czk) treated seedlings (control) after 1 week of incubation.

Identification of Potent Fungal Isolate

Khan, et al. [24] 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 blasted using BLASTn1 software for sequence similarity estimate. Phylogenetic tree was constructed with NJ (Neighbor Joining) via MEGA-7 Software.

Fungal Inoculation of *H. annuus* and *G. max*

Fresh biomass of *G. Cobalt* was applied to the pots containing sterilized sand. Six seeds of each *G. max* or *H. annuus* were placed in these pots and the pots were carried to the growth chambers, running at 25ºC or 40ºC. Hoagland solution was applied as a nutrient source. After two weeks of fungal inoculation growth characteristics (root and shoot lengths, root and shoot fresh weights and root and shoot dry weights) of endophyte-aligned and endophyte-free *G. max* and *H. annuus* were recorded [25]. The experiment was accomplished in triplicates.

Determination of Phenolics and Proline

Phenolics in *G. max* and *H. annuus* were determined via folin-ciocalteu colorimetric method [26]. Analytical grade gallic acid (100, 200, 300, 500, 600, 700, and 900 mg/ml) was used to construct a standard curve with optical density taken at 650 nm. Methodology of Bates,

et al. [27] was used with minor modifications for the investigations of total proline. Proline (Sigma Aldrich; 2, 4, 6, 8, and 10 µg/ml) was used to construct a standard curve with optical density recorded at 520 nm.

Determination of Antioxidants

For the determination of antioxidants fresh leaves of *G. max* and *H. annuus* (10 g) were drenched in sodium-phosphate buffer (50 mM, pH 7), containing 1% soluble polyvinyl-pyrolidine (w/v). The mixture was then rotated at 15,000 rpm at 4ºC for twenty minutes. Supernatant was used to analyze enzymatic activity. Catalase (EC 1.11.1.6) activity was observed using Luck [28] procedure with minor modifications. Extinction co-efficient (36×10^3 mM^{-1} cm^{-1}) was applied to measure CAT activity and was shown as EU mg^{-1} protein. Methodology of Kar and Mishra [29] was used for POD, EC 1.11.1.7) activity. The reaction mixture contained H_2O_2 (50 µM), H_3PO_4 buffer (125 µM pH 6.8), pyrogallol (50 µM) and 20X diluted enzyme extract (1 ml). The production of purpurogallin was observed at 420 nm. POD concentration was calculated as EU mg^{-1} protein. Beyer Jr and Fridovich [30] procedure was followed for the examination of SOD (EC 1.15.1.1) activity by observing a reduction in the OD of nitro-blue tetrazolium (NBT). Quantification of SOD was examined as enzyme unit (EU) mg^{-1} protein and 1 unit of enzyme was considered as the measure of protein starting a 50% inhibition of NBT reduction. Carlberg and Mannervik [31] methodology was used for the activity of GR (GR, EC 1.6.4.2). GR was measured by reduction in OD at 340 nm for two minutes. GR concentration was measured by applying an extinction co-efficient of 0.12 mM NADPH, which was 6.2 mM^{-1} cm^{-1}, and was shown as EU mg^{-1} protein.

Determination of Total Soluble Sugars, Lipid and Proteins

Mohammadkhani and Heidari [32] protocol was used for the investigation of total soluble sugars. Different concentrations of glucose were used for plotting standard curve (20, 40, 60, 80 and 100 µg/ml) and OD was recorded at 485 nm. Van Handel [33] methodology was adopted for the analysis of lipids with minor modifications. Various concentrations of canola oil were used to plot standard curve (10, 40, 70, 100, 130 and 160 µg/ml) and OD was taken at 490 nm. Methodology of Lowry, et al. [34] was followed for the investigation of total proteins. Different concentrations of BSA (20, 40, 60, 80 and 100 µg/ml) were applied to plot a standard curve. Optical density was taken at 650 nm.

Determination of ABA

Abscisic acid in the leaves of *G. max* and *H. annuus* was determined via optimized protocol of Yoon, et al. [13]. Fresh leaves of *G. max* and *H. annuus*, half gram each, were grinded in pestle and mortar using liquid N_2, followed by the addition of glacial acetic acid (28.5 ml) and isopropanol (1.5 ml). Sample was then filtered through filter paper and dehydrated with the help of rotary evaporator. Diazo-methane was added to this mixture and then observed via GC–MS SIM (6890N network GC system furnished with 5973 network mass-selective-detector, Agilent Technologies, Palo Alto, CA, USA). The Lab-Base (Thermo Quset, Manchester, UK) data system software was applied to detect retorts to ions with m/z values of 162 and 190 for Me-ABA and 166 and 194 for Me-[2H6]-ABA. For internal standard, ABA ([2H6]-ABA) (Sigma Aldrich) was used.

Statistical Analysis

A complete randomized design with one way ANOVA was applied to analyze the data. The means of all values were equated by DMRT (Duncan Multiple Range Test) at $p<0.05$, via SPSS-20 (SPSS Inch., Chicago, IL, USA). All the experiments were done in triplicates.

Table 1. Effect of *G. cibotii* filtrate on the growth of rice seedlings.

Growth attributes	Control (DW)	Control (Czk)	G. *cibotii*
Shoot Length (cm)	10.4±.8[a]	10.6±1.5[a]	12.6±1.4[a]
Root Length (cm)	4.9±.8[a]	6.3±.3[a]	7.6±1.2[a]
Fresh Weight Shoot (g)	0.03±.02[a]	0.0371±.03[a]	0.0383±.022[a]
Fresh Weight Root (g)	0.08±.07[a]	0.095±.05[a]	0.105±.01[a]
Dry Weight Shoot (g)	0.0047±.03[a]	0.0043±.03[a]	0.0053±.03[a]
Dry Weight Root (g)	0.0137±.08[a]	0.015±.01[a]	0.017±.01[a]
Chlorophyll (SPAD)	21.4±0.8[a]	24±0.6[b]	27±0.6[c]

Czk = Czapek medium, DW = Distilled water. Data are means of 3 replicates with standard error. The data in each row followed by different letters are significantly different ($p<0.05$) from each other as estimated by Duncan's Multiple Range Test (DMRT).

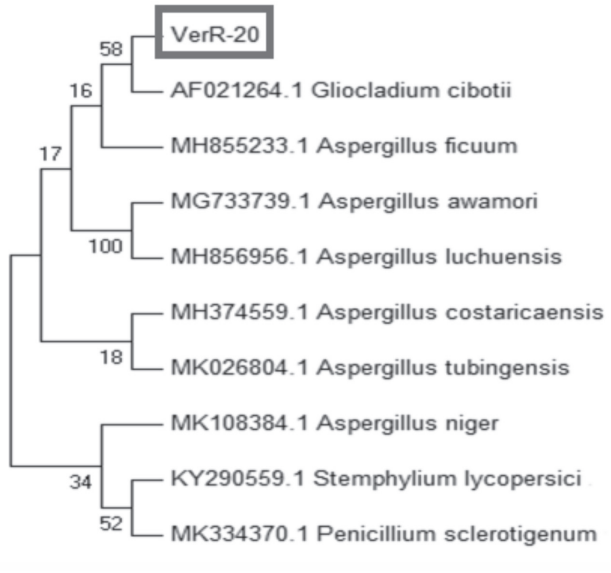

Fig. 1. Phylogenetic consensus tree construction (using 10 taxa, 9 reference and 1 clone) for the identification of fungal isolate VerR-20 using neighbor joining (NJ) method. 58% bootstrap value confirmed isolate VerR-20 as *Gliocladium cibotii*.

Results and Discussion

Isolation and Plant Growth Promoting Activity of Endophytic Fungi

Five different fungal isolates were collected from *V. officinalis* L. and their potential for plant growth promotion was checked on *O. sativa*. Enhanced growth features [chlorophyll contents (12.5%), shoot length (20%), root length (21%), shoot fresh weight (3%), root fresh weight (10%), shoot dry weight (23%) and root dry weight (13%)] were recorded in VerR-20-treated *O. sativa* seedlings as compared to Czapek-treated control (Table 1). Fungal isolate VerR-20 significantly enhanced growth of *O. Stevia* and were selected for further identification and analysis.

Molecular Identification of Isolate VerR-20

18S rDNA nucleotide sequence of the Internal Transcribed Spacer (ITS region) of the isolate VerR-20 was compared with allied strains by applying BLAST search program (Basic Local Alignment Search Tool, 2012). Nucleotide sequence showed maximum resemblance (58%) with *G. cibotii*. Phylogenetic tree was constructed from 10 (1 cloned and 9 references) via NJ (Neighbor Joining) technique using MEGA-7.0 software (Fig. 1). Results of phylogenetic examination and sequence homology suggested isolate VerR-20 as *Gliocladium cibotii.*

Growth Features of *G. max* and *H. annuus* Co-Cultured with *G. cibotii*

G. max and *H. annuus* were incubated for two weeks at normal and high temperatures. The growth traits of *G. cibotii*-associated *G. max* and *H. annuus* plants were measured and equated with non-associated ones. A significant variation was recorded in all growth traits in *G. max* and *H. annuus* seedlings allied with *G. cibotii* as compared to control seedlings. Endophyte-associated *G. max* has enhanced chlorophyll (8%), shoot length (20%), root length (9%), fresh shoot (26%) and root weights (31%) and shoot (38%) and root dry weights (58%), when exposed to thermal stress as equated to non-aligned seedlings (Table 2). Likewise, a rise was also noted in the shoot lengths (15.5%), root lengths (52%), fresh shoot weight (33.5%), fresh root weight (47.6%), dry shoot weight 16%), dry root weight (24%) and chlorophyll content (11.4%) of *H. annuus* plantlets co-cultured with *G. cibotii* at high temperature than endophyte-free plants (Table 3). We detected a high content of chlorophyll in *G. cibotii*-inoculated *G. max* and *H. annuus* as compared to control seedlings, which might be due to enhancement in the uptake of Mg element by *G. cibotii* [2]. In a likewise study, a high rate of photosynthesis was recorded in *Capsicum annuum* colonized by an endophytic fungi *Penicillium*

Table 2. Effect of *G. cibotii* on the growth features of *G. max*.

Growth attributes/ temperature stress	25ºC		40ºC	
	Control	*G. cibotii*	Control	*G. cibotii*
Total Chlorophyll (SPAD)	$31.6+0.2^{ab}$	$37+1.4^{b}$	$28+2^{a}$	$30.3+.6^{ab}$
Shoot Length (cm)	$37.7+2.8^{c}$	$40.3+1.9^{c}$	$25+0.8^{a}$	$30+1.2^{b}$
Root Length (cm)	$15.4+1.2^{ab}$	$16+0.6^{ab}$	$11+0.5^{a}$	$12+1^{a}$
Fresh Weight Shoot (g)	$1.44+0.05^{ab}$	$1.51+0.14^{b}$	$0.81+0.45^{a}$	$1.02+0.3^{ab}$
Fresh Weight Root (g)	$0.187+0.06^{a}$	$0.238+0.03^{a}$	$0.131+0.04^{a}$	$0.172+0.3^{a}$
Dry Weight Shoot (g)	$0.136+0.02^{b}$	$0.169+0.03^{c}$	$0.081+0.02^{a}$	$0.112+0.5^{ab}$
Dry Weight Root (g)	$0.081+0.04^{c}$	$0.092+0.03^{d}$	$0.012+0.02^{a}$	$0.019+0.3^{b}$

The data presented are mean of 3 replicates with standard error. The data in each row followed by different letters are significantly different ($p<0.05$) from each other as estimated by Duncan's Multiple Range Test (DMRT).

Table 3. Effect of *G. cibotii* on the growth features of *H. annuus.*

Growth attributes/ temperature stress	25ºC		40ºC	
	Control	*G. cibotii*	Control	*G. cibotii*
Total Chlorophyll (SPAD)	39.4+1.5[a]	44.5+1.3[a]	33.2+1.7[a]	37+1.6[a]
Shoot Length (cm)	25.6+1.8[a]	26+1.4[a]	21.3+0.3[a]	24.6+0.8[a]
Root Length (cm)	9.2+1.3[ab]	10+1.3[b]	4.6+0.4[a]	7+1.3[b]
Fresh Weight Shoot (g)	1.31+0.04[b]	1.39+0.5[b]	0.81+0.7[a]	1.082+0.05[a]
Fresh Weight Root (g)	0.152+0.04[ab]	0.192+0.03[b]	0.082+0.06[a]	0.121+0.02[ab]
Dry Weight Shoot (g)	0.092+0.07[b]	0.099+0.08[b]	0.05+0.07[a]	0.058+0.04[a]
Dry Weight Root (g)	0.031+0.02[b]	0.039+0.05[c]	0.021+0.02[a]	0.026+0.01[a]

The data presented are mean of 3 replicates with standard error. The data in each row followed by different letters are significantly different ($p<0.05$) from each other as estimated by Duncan's Multiple Range Test (DMRT).

resedanum [35] and *Chaetomium globosum* [36] under the scarcity of water. Enhancement in the rate of photosynthesis is accompanying with high contents of chlorophyll as well as increase in surface area of leaves in endophyte-colonized seedlings as equated to non-colonized plants [37]. An endophytic fungus, *Piriformospora indica*, was also known to be growth supporter of *Arabidopsis* that increased total chlorophyll

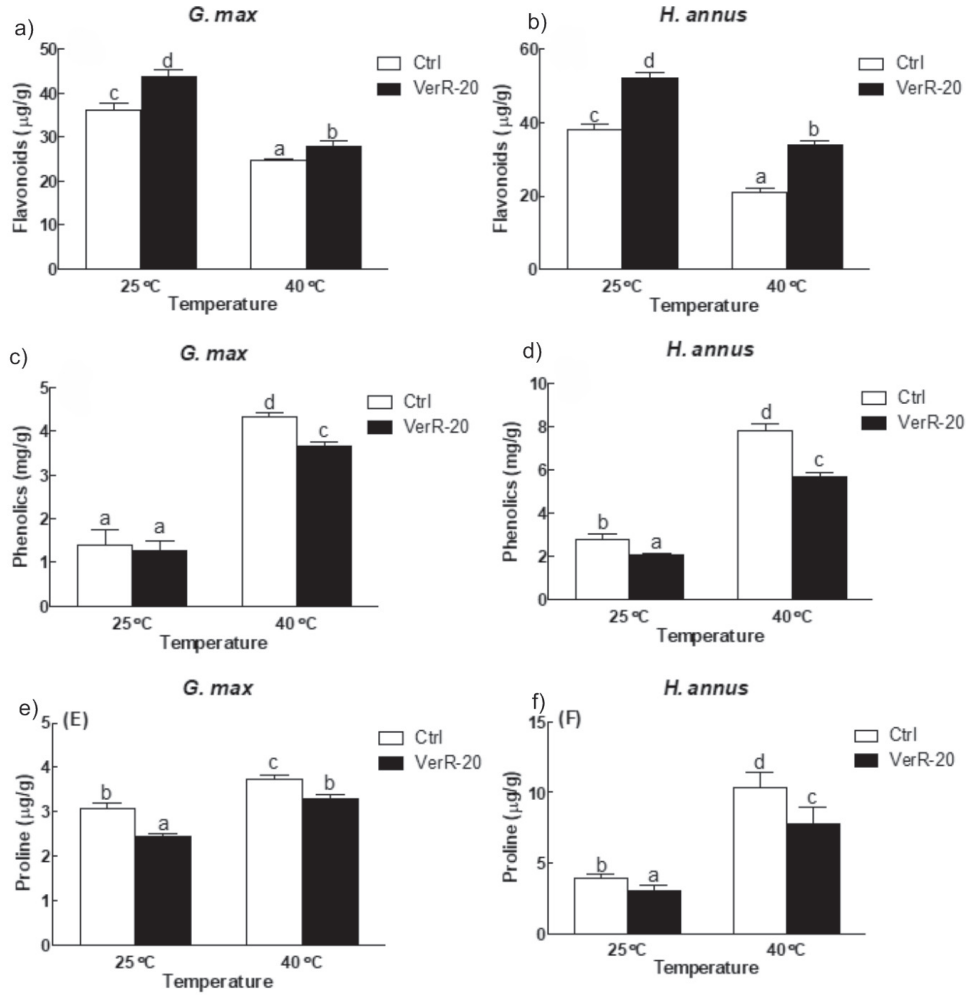

Fig. 2. a) Flavonoids, b) phenolics and c) proline contents of *G. max* and *H. annuus* inoculated with and without *G. cibotii*. Data are means of 3 replicates with standard error. Different letters are significantly different ($p<0.05$) as estimated by Duncan's Multiple Range Test (DMRT).

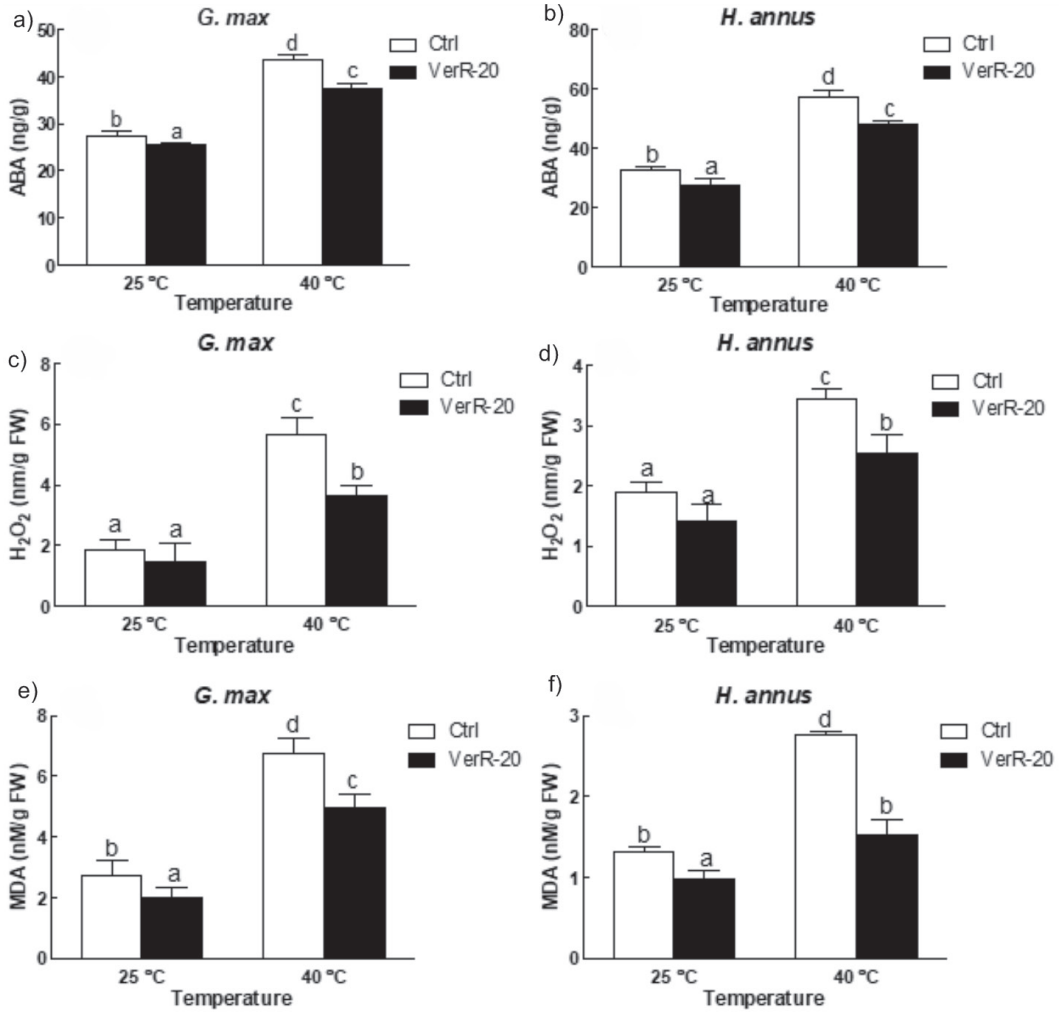

Fig. 3. a) ABA b) Hydrogen peroxide (H_2O_2) and Lipid peroxidation (MDA) in *G. max* and *H. annuus* inoculated with and without *G. cibotii*. Data are means of 3 replicates with standard error. Different letters are significantly different ($p<0.05$) as estimated by Duncan's Multiple Range Test (DMRT).

contents and hence rate of photosynthesis under abiotic stress [38]. Endophytes help in maintaining rate of photosynthetic in their host plants by protecting their thylakoid-membrane-proteins and chlorophyll molecules from denaturation by abiotic stresses [39].

Mediation of Physiological Attributes

Maximum increase (36.8%) in the levels of flavonoids and phenolics was recorded in the seedlings of *H. annuus*, whereas *G. cibotii* was less responsive in *G. max* (4%) seedlings in terms of rise in phenolics concentrations as equated to fungal-free seedlings (Fig. 2a-b). We observed increased level of phenolic contents in *G. max* and *H. annuus* while, a decrease was noticed in the level of proline contents under heat stress, when co-cultured with *G. cibotii* as compared to the control plants (Fig. 2c). Phenolic compounds play defensive role in higher plants against biotic and abiotic stresses and their concentrations exceeds than the normal level under various ecological constrains [40]. Endophyte-inoculated plants have high concentration of phenolics than non-inoculated ones under abiotic stresses. The accumulation of high content of phenolics in endophyte-inoculated *G. max* and *H. annuus* plants under thermal stress help in mitigating the accretion of ROS in stressed plants. Our study is in line with the work of Abd_Allah, et al. [41] that endophytes boost phenolic levels of the host plant under abiotic stress. Proline accumulates in various plants in response to different environmental stresses, including high temperature and is known to function as organic osmolyte.

A decline was also observed in the quantity of ABA in the leaves of *G. cibotii*-associated *G. max* and *H. annuus* seedlings at 25ºC and 40ºC as equated to non-aligned plantlets. *G. max* seedlings co-cultured with *G. cibotii* has 7% lesser ABA as equated to control seedlings at 25ºC, while at 40ºC, endophyte-inoculated *G. max* has 16% lower ABA as equated to *G. cibotii*-free seedlings. Similarly, *G. cibotii*-inoculated *H. annuus* has 17.9% lesser ABA content at 25ºC, while 18% lesser

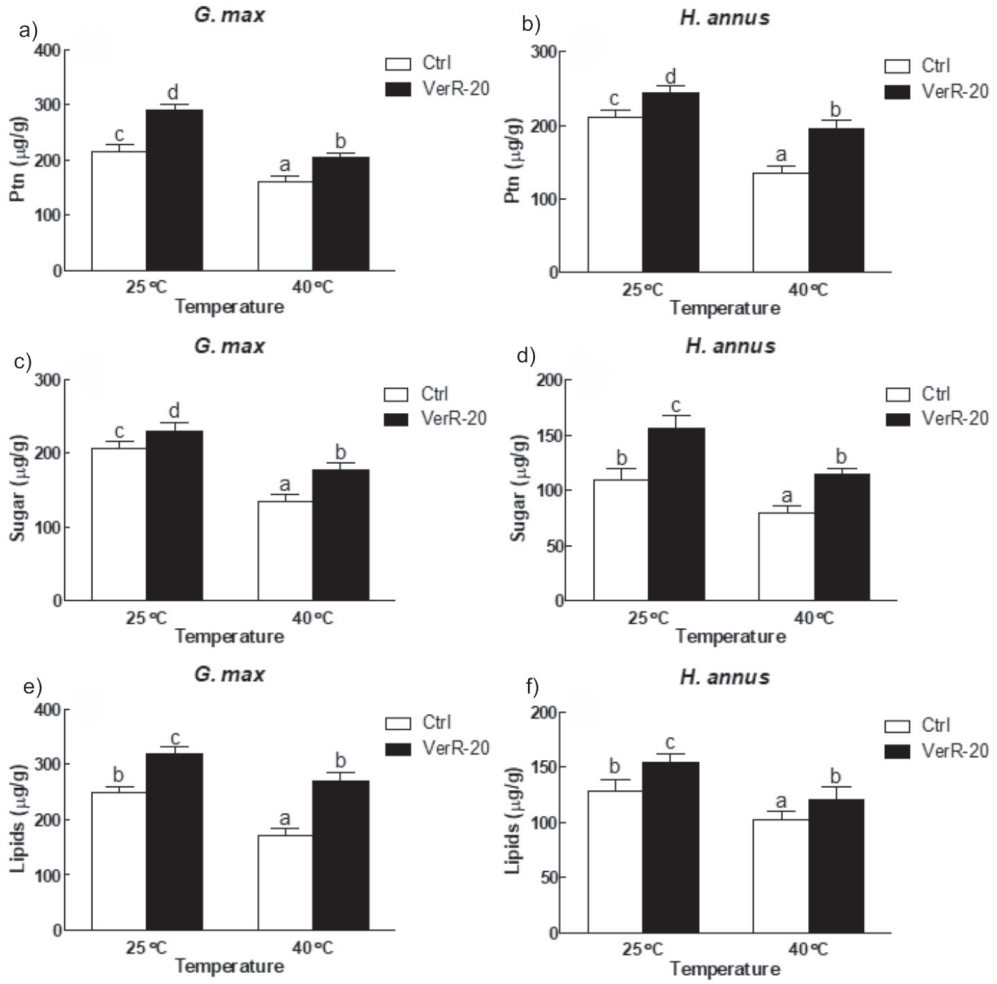

Fig. 4. Analysis of total proteins a), soluble sugars b) and lipids c) of *G. max* and *H. annuus* inoculated with *G. cibotii*. Data are means of 3 replicates with standard error. Different letters are significantly different ($p<0.05$) as estimated by Duncan's Multiple Range Test (DMRT).

ABA content at 40ºC as compared to endophyte-free plants (Fig. 3a). High concentration of H_2O_2 and lipids peroxidation in control *G. max* and *H. annuus* seedlings exposed to high temperature. At 25ºC, *G. cibotii* abridged H_2O_2 levels were found in *G. max* (25%) and *H. annuus* (32%), while at 40 ˚C this decline was 56% and 34%, respectively in the tested species (Fig. 3b). Concentration of lipids peroxidation was premeditated in relations to MDA production. A substantial decline was recorded in the quantity of MDA in *G. cibotii*-aligned *G. max* and *H. annuus*. At 25ºC this decrease in the amount of MDA was 39.6% and 36.7% in the

Table 4. Effect of *G. cibotii* on the ativity of antioxidant enzymes in *G. max.*

Enzyme/temperature stress	25ºC		40ºC	
	Control	*G. cibotii*	Control	*G. cibotii*
AAO (Unit/g/30 sec.)	1.49± 0.07^a	1.58±0.03^b	1.82±0.09^c	1.98+0.09^d
CAT (Unit/g/30 sec.)	0.42±0.05^a	0.55±0.02^b	0.91+0.5^c	1.01+0.07^d
POD (U/mg protein)	1.44±0.04^a	1.57+0.08^a	3.69+0.29^b	4.89+0.91^c
SOD (U/mg protein)	19±0.91^a	18.2±0.95^a	35±0.9^b	40±1.9^c
GR (U/mg protein)	1.60± 0.59^a	0.157±0.14^a	1.76±0.08^b	2.01±0.14^c

AAO = ascorbic acid oxidase; CAT = catalase; POD = peroxidase, SOD = superoxide dismutase; GR = glutathione reductase.
The data presented are mean of 3 replicates with standard error. The data in each row followed by different letters are significantly different ($p<0.05$) from each other as estimated by Duncan's Multiple Range Test (DMRT).

Table 5. Effect of *G. cibotii* on the activity of antioxidant enzymes in *H. annuus*.

Enzyme/temperature stress	25ºC		40ºC	
	Control	*G. cibotii*	Control	*G. cibotii*
AAO (Unit/g/30 sec.)	0.68± 0.09[a]	0.71±0.01[b]	2.18±0.36[c]	2.48+0.31[c]
CAT (Unit/g/30 sec.)	0.24±0.05[a]	0.30±0.06[b]	0.63+0.09[c]	0.70+0.08[d]
POD (U/mg protein)	1.21±0.05[a]	1.34+0.11[a]	2.75+0.19[b]	3.39+0.10[c]
SOD (U/mg protein)	12.8±0.32[a]	12.9±0.24[a]	22.8±1.08[b]	24.9±0.92[c]
GR (U/mg protein)	0.85± 0.06[a]	0.91±0.06[a]	2.18±0.08[b]	2.56±0.11[c]

AAO = ascorbic acid oxidase; CAT = catalase; POD = peroxidase, SOD = superoxide dismutase; GR = glutathione reductase.
The data presented are mean of 3 replicates with standard error. The data in each row followed by different letters are significantly different ($p<0.05$) from each other as estimated by Duncan's Multiple Range Test (DMRT).

G. max and *H. annuus* seedlings respectively, while at 40ºC the decrease was more in *H. annuus* (79%) than in *G. max* (35.7%) seedlings (Fig. 3c). Plants are known to initiate some complicated biosynthetic responses, like adjustments in membrane lipids, antioxidant systems, osmotic potential and synthesis of heat-shock-proteins exposed to thermal stress [22]. In the present study, we noticed more resistance in *G. cibotii*-inoculated *G. max* and *H. annuus* seedlings subjected to heat stress than uninoculated seedlings. High temperature disturbs plant´s physiological, biochemical and transcriptional mechanisms. Elevated level of MDA in endophyte-free plants is due to oxidative stress. High concentrations of MDA may leads to membrane damages. The endophytes, including bacteria and fungi are known to reduce the impacts of abiotic stresses by reducing the level of MDA in their host plants. In this study, we observed high concentrations of MDA in both *G. max* and *H. annuus* control seedlings subjected to heat stress as compared to *G. cibotii*-associated plants. In a similar type of study, 27.5% decline in the amount of MDA was recorded in chickpea plants co-cultured with *Bacillus subtilis* BERA 71 subjected to 200 mM NaCl salt stress [41]. Our findings confirmed the study of Abd_Allah, et al. [41], who observed that endophytes ameliorate abiotic traumas by reducing the concentration of H_2O_2 and MDA. Moreover, high temperature also enhances H_2O_2 in plants, which ultimately affect membrane structural integrity and directs lipids peroxidation and hence more MDA synthesis as indicated in control *G. max* and *H. annuus* seedlings [2]. Abscisic acid, a plant stress hormone, gathers in higher plants under abiotic stresses, including high temperature. Higher amount of ABA was detected in *O. sativa* plants exposed to high temperature [42]. Heat stress results in down-regulation of those genes which are responsible for ABA catabolism through up-regulation of ABA biosynthesis genes [43]. In the current study, we noticed a small amount of ABA in soybean and sunflower inoculated with *G. cibotii* imperiled to high temperature stress as compared to un-inoculated seedlings. This drop in ABA concentration in experimental plants might be owing to down regulation of genes responsible for ABA synthesis. Decline in ABA level in fungal-endophyte allied *G. max* and *H. annuus* confirmed the discovery of Hamayun, et al. [6]. Low concentration of ABA in endophyte-allied plantlets might be due to the involvement of GAs, as exogenous application of GAs enhanced stress tolerance in *G. max* escorted by reduced amount of ABA [1].

A considerable increase in the activities of antioxidants was noticed, including AAO, CAT, POD, SOD and GR in endophyte-aligned *G. max* and *H. annuus* seedlings at 40ºC as equated to non-aligned *G. max* seedlings. *G. cibotii*-aligned *G. max* plantlets had improved concentrations of CAT (13%), POD (39%), AAO (2%), SOD (27%) and GR (25%). Similarly, the *G. cibotii*-associated *H. annuus* had mended levels of CAT (43%) POD (37%), AAO (28%), SOD (22%) and GR (36%) as compared to endophyte-free seedlings (Tables 4-5). High concentration of proline in *G. cibotii*-associated *G. max* and *H. annuus* had encouraging effects on enzymes, free radicals scavenging, buffering of cellular redox potential, membrane integrity as well as in mediating osmotic modification in plants under stressful environment [19]. Endophytic fungus *G. cibotii* displayed an ameliorative role against heat stress that might be due to the up-regulation of nutrients uptake and antioxidant system. Moreover, *G. cibotii* helped in declination of ROS. CAT, POD, SOD, AAO and GR of host plants. The up-regulation of the antioxidants system can protect membranes of plants from the perilous effects of free radicals generated during stress conditions [41].

Total soluble sugars, lipids and proteins of *G. max* and *H. annuus* were determined via spectrophotometer. At 40ºC, *G. cibotii*-aligned *G. max* had 31% and *H. annuus* had 24% more soluble sugars, while at 25ºC, *G. max* had 27% and *H. annuus* had 16% more soluble sugars as equated to *G. cibotii*-free seedlings (Fig. 4a). A rise of 28% and 14% was recorded in the total lipid contents of *G. cibotii*-aligned *G. max* and *H. annuus* plantlets, at 25ºC as compared to uninoculated plants. The highest concentration of lipids was observed in *G. cibotii*-associated *G. max* (25%) and *H. annuus* (18%) as equated to control seedlings

at 40ºC (Fig. 4b). Furthermore, endophyte-aligned *G. max* had 21% and *H. annuus* had 14% more proteins at 40ºC, while at 25º**C**, *G. max* had10% and *H. annuus* had 6% more proteins as compared to endophyte-free plants (Fig. 4c). Higher nutritious value, such as total sugars, lipids and proteins of *G. cibotii*-associated *G. max* and *H. annuus* was found under thermal stress indicating the encouraging role of endophytic fungi in crops production. Additionally, the results suggesting the usage of *G. cibotii* as bio-fertilizers. Rodriguez and Redman [44] also point out the protective role of *A. flavus* for host plant against heat stress.

Conclusions

Heat stress is considered as one of the major ecological disorders that depresses the quality as well as the quantity of food crops, including *G. max* and *H. annuus*. The current study verified the thermo-tolerant potential of *G. cibotii*. The endophyte *G. cibotii* not only improved growth features in *G. max* and *H. annuus*, but also boosted ROS scavenging aptitude of host plants, like CAT, POD, SOD, AAO, GR, proline and phenolics against thermal stress. Endogenous concentration of ABA, lipids, proteins and sugars in *G. max* and *H. annuus* were improved significantly. The results recommend the use of *G. cibotii* as heat stress alleviating bio-agent and bio-fertilizer in the future for sustainable agriculture.

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Conflict of Interest

The authors declare no conflict of interest.

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