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Evaluating Plant Growth Promoting and drought Stress Alleviating Traits in Fungal Endophytes

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ABSTRACT: Drought is one of the devastating environmental stresses which has adverse effect on yield and productivity in all agricultural crops. Among different beneficial microorganisms, fungal endophytes are known impart drought tolerance through physiological, biological and biochemical modifications in plants. They secrete metabolites that combat stress or stimulate the early stress response system in host plant to cope with the stress. The current study aims to characterize, six fungal endophytes *viz*., P1R1, P4L5, P6R3, P6R4, P10R1 and P12R2 with respect to plant growth promotion and drought stress alleviating traits under *invitro* conditions. The results showed that all six fungal endophytes were able to solubilize phosphate and produced phytohormones while two fungal endophytes showed siderophore production. Fungal endophyte P6R4 showed the highest phosphate solubilization index and isolate P10R1 showed high siderophore producing index. Stress induced IAA and GA production was observed to be maximum in isolates P10R1 and P6R4 respectively. Similarly, the production of high phenol and flavonoids in their culture filtrates indicated their antioxidant and reducing power activity under stress conditions among the isolates. The study reveals that fungal isolates have the ability to impart drought tolerance in host plants through plant growth promoting activities.

Keywords: Fungal endophytes, drought, phytohormones, antioxidant activity.

INTRODUCTION

In arid and semi-arid regions, drought has adversely affected the plant growth, development, yield, quality, and productivity in all agricultural crops posing serious threat to the global food security (Seleiman *et al*., 2021; Diatta *et al.,* 2020; Okorie *et al.,* 2019). It has been reported that, during the years 1980-2015 drought resulted in 40% and 21% yield reduction in maize and wheat respectively (Daryanto *et al*., 2016). Also, combined effects of high temperatures and drought significantly decreased yield of maize, soybeans, and wheat by 11.6, 12.4, and 9.2%, respectively globally (Matiu *et al*., 2017). To combat drought stress and boost crop output to fulfil the growing nutritional demands of the global population through sustainable agriculture, many drought mitigation measures have been developed. One such strategies include use of beneficial microorganisms such as plant growth-promoting rhizobacteria (PGPR), mycorrhizae, and fungal endophytes (Yasmin *et al*., 2017; Bahadur *et al*., 2019; Kamran *et al.*, 2022).

Fungal endophytes are the eukaryotes that colonize plant tissues intercellularly and/ or intracellularly without causing any disease or showing any visual symptoms. They are known to mediated plant growth and development under drought conditions through various mechanisms that are either direct or indirect. The phytohormone production by endophytes is the principal mechanism that enhances plant growth during which physiological and morphological changes are observed in plants (Adeleke and Babalola 2021). Phytohormones modulate the developmental processes and signaling networks in host plants which aid in acclimating plants to abiotic stresses, also their survival (Wahab *et al.*, 2022). The most prevailing phytohormones produced by fungal endophytes are auxins, cytokinin, gibberellin (GAs), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA). Among these auxins, GAs and cytokinin are developmental hormones and SA, ABA and JA are stress-related hormones (Cosoveanu *et al*., 2021). Naureen *et al.* (2022) revealed that the root-endophytic fungus CJAN1179 from *Cymbopogon jwarancusa* produced indole-3-acetic acid (IAA) particularly in the presence of tryptophan. Waqas *et al.* (2012) showed increased salt and drought stress tolerance in cucumber plants by GA-producing endophytic fungus *Phoma glomerata* LWL2 LWL3.

Plant growth promotion is also eased by nutrient acquisition by fungal endophytes. They mobilize insoluble phosphate and provide nitrogen to their host

plant (Hiruma *et al.*, 2018; Hassan, 2017). The soil nutrients like iron, phosphorus, nitrogen, and potassium are made available to plants through mineralization of elements by fungal endophytes (Adeleke and Babalola 2021). Adhikari and Pandey (2019) showed the potential of endophytic fungi (*Penicillium* and *Aspergillus* spp.) isolated from roots of *Taxus wallichiana*, for their ability to solubilize insoluble phosphates in presence of tricalcium (TCP), aluminium (AlP), and iron phosphate (FeP) at different temperatures through production of phosphatases, phytases and organic acids. Dark septate fungal endophyte (*Ophiosphaerella* sp., *Cochliobolus* sp., *Drechslera* sp., *Curvularia* spp.) isolated from the roots of wheat (*Triticum aestivum*) has potential to solubilize calcium, aluminium, and iron phosphates under *invitro* conditions (Spagnoletti *et al.*, 2017).

Fungal endophytes protect the plant from drought mediated osmotic stress through the production of various osmo-protectants such as water-soluble sugars, amino acids like proline, fungal metabolites like mannitol and trehalose, glycine betaine *etc* (Ahmad *et al.*, 2016; Nagabhyru *et al.*, 2013). When fungi are exposed to water stress, they also accumulate compatible solutes that aid in osmotic adjustment or as a desiccation protectant in mycelia or conidia (Jennings and Burke 1990). Compatible solutes include sugars (glucose, glycerol, trehalose) and sugar alcohols (sorbitol, mannitol, erythritol) that are accumulated in mycelial at initial growth phase and then transported to conidia in the later stages of the fungal growth (Ramirez *et al.*, 2004). Trehalose is a nonreducing disaccharide of glucose synthesized by fungi that serves as a reserve carbohydrate as well as a stress protectant (Singer and Lindquist 1998; Thevelein, 1984).

Drought stress cause excess synthesis of reactive oxygen species (ROS) causing oxidative stress and damaging proteins, lipids and DNA (Gill and Tuteja 2010). Fungal endophytes either directly scavenge ROS by producing ROS scavenging compounds or induces plants to do or they prevent the production of ROS when plant is exposed to abiotic stresses (Ma *et al.*, 2019). Srinivasan *et al.* (2010) reported phenolic and flavonoid content in **e**ndophytic fungus culture extract and showed activity of against ABTS and DPPH radicals.

Endophytes of plants from different extreme habitats, such as geothermal soils and coastal confer habitat-specific stress tolerance to plants through habitat-adapted symbiosis (Rodriguez *et al.*, 2008). In some cases, the plants are unable to survive in their native stress habitats without the habitat-adapted fungal endophytes (Rodriguez and Redman 2008). This phenomenon is unique to Class 2 endophytes where individual isolates colonize asymptomatically and confer habitat-adapted fitness benefits on genetically distant host species representing monocots and eudicots (Rodriguez *et al.*, 2009). Researchers are investigating new endophytes from extreme plants growing in extreme climate for imparting drought tolerance in plants. In the previous study fungal endophytes were isolated form drought adapted plants growing in arid and semi-arid regions of Karnataka and screened for imparting drought tolerance in maize seedling (Shetty and Earanna 2023). The present study is focused on evaluating the plant growth promoting and drought alleviating characteristics of fungal endophytes.

MATERIALS AND METHOD

Fungal cultures. Six fungal endophytes *viz.* P1R1, P4L5, P6R3, P6R4, P10R1 and P12R2 were used this study which was previously screened for imparting drought tolerance in maize (Shetty and Earanna 2023). Fungal endophytes were cultured on potato dextrose agar (PDA) media supplemented with 100 µg/ mL ampicillin and grown at 30ºC.

Determination of phytohormones in fungal filtrate using High Performance Liquid Chromatogram (HPLC). Endophytic fungi were inoculated on potato dextrose agar medium for 7 days at 30ºC. One disc (5 mm) of each fungal culture was inoculated in each 100 mL of PDB and PEG amended PDB (-10.31MPa) and incubated at 30ºC for 14 days. Uninoculated medium was used as control. After 14 days, the culture filtrate of each flask was passed through Whatman No. 42 filter paper. The pH of filtrate was adjusted to 2.5 by adding 0.1 N HCl or KOH. Extraction was done as per the method described by Rachev *et al.* (1993). The culture filtrate was extracted three times using ethyl acetate in separating funnel. The organic layer was separated and passed through anhydrous sodium sulphate. The solvent was evaporated in a rotary vacuum evaporator at 40ºC and 10 rpm (Hasan *et al.*, 2002). The residue was dissolved in HPLC-grade methanol. The HPLC analyses was carried out on a Shimadzu instrument (Prominence–I, LC-2030C) equipped with a UV detector (LC-2030 UV detector) and fitted with a C_{18} reverse phase HPLC column (Shim-pack GIST C_{18}, Dimension 250 × 4.6 mm, particle size 5 µm). The column temperature, 30ºC was maintained for all the samples with other specific conditions described below.

Phytohormone	Solvent	Wavelength (nm)	Flow rate (mL/min)
IAA	Methanol: water (80:20)	270	1.0
GA	Methanol: water (70:30)	208	0.8
SA	Acetonitrile: Acetic acid 0.5 % (90:10)	302	1.0
ABA	Acetonitrile: Acetic acid 0.5 % (80:20)	254	0.8

Phosphate, Potassium solubilization and Siderophore production. The phosphate solubilizing potential of fungal endophytes was evaluated *in- vitro* as described by Doilom *et al.* (2020). The ability of potassium solubilization by fungal endophytes was determined by growing on Aleksandrow agar medium (Hu *et al.*, 2006). The diameter (mm) of clear zones around fungal plugs was measured. The siderophore

production was assessed by placing 5 mm freshly grown mycelial discs on Chrome Azurol S (CAS) solid medium (Schwyn and Neilands 1987). The diameter of the colony and orange colour zone surrounding the colony was measured. The solubilization index or Siderophore producing index (SPI) was calculated according to the formula below.

Solubilization Index (SI) =

$$\frac{\text{Colony diameter + Clearing zone diameter}}{\text{Colony diameter}}$$

Determination of total phenolic and flavonoid contents. Endophytic fungi were grown on PDA agar for 7 days at 30ºC. One disc (5 mm) of each fungal culture was inoculated in 100 mL of PDA broth and PEG amended PDB, then incubated at 30ºC for 14 days. After 14 days, mycelium was separated by filtration using sterile filter paper. The filtrate was extracted three times in ethyl acetate using separatory funnels and concentrated using a rotary evaporator until dried (Salini *et al.*, 2015). The dried extract was dissolved in ethanol and stored at -20 ºC for estimation of phenols and flavonoids.

The total phenol content of fungal extract was estimated using the Folin–Ciocalteu reagent method described by Hameed *et al.* (2017). Fungal extracts (250 µL) were mixed with 250 µL of Folin- Ciocalteu reagent and incubated for 2 minutes. Then, 500 µL saturated sodium carbonate (10%, w/v aqueous solution) was added and incubated in dark for 1h. The reaction mixture was measured at 765 nm using spectrophotometer (UV-VIS, Systronics Ltd., India). The concentration of total phenols was calculated based on a calibration curve using gallic acid. Total phenolic content was expressed as mg of gallic acid equivalent per gram of dried extract (mg GAE/ g dry weight).

The total flavonoid content of fungal extract was estimated by the method described by Hameed *et al.* (2017). Fungal extract (0.5 mL) was mixed with 1.0 mL of a 2% (v/v) $AlCl_3.6H_2O$ ethanolic solution and incubated for 10 minutes. The absorbance was measured at 430 nm using spectrophotometer (UV-VIS, Systronics Ltd., India). The concentration of total flavonoid was calculated based on a calibration curve using quercetin. Total flavonoid content was expressed as mg quercetin equivalent to per gram of dried extract.

Antioxidant assay

$ABTS^+$ scavenging activity. The antioxidant activity assay was performed by free radical scavenging method using ABTS [2, 2'-azino-bis (3-ethyl-benzthiazolin-6-sulfonicacid)] as described by Hameed *et al. (*2017). A stock solution of ABTS (7 mM) was prepared. ABTS radical cations (ABTS+) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (1:1) and incubated in dark for 16 h. ABTS+ solution was diluted in 95% ethanol to an absorbance 0.75 at 734 nm. Endophytic fungal extracts (20 µL) were added to 180 µL of diluted $ABTS^+$ solution and incubated for 2 minutes at room temperature. The scavenging activity of the fungal extracts was assessed from the percentage of decolorization at 734 nm using spectrophotometer (UV-VIS, Systronics Ltd., India). The $ABTS^+$ scavenging activity (%) was calculated using the equation as follows:

$ABTS^+$ scavenging activity = $[(OD_{734control}-OD_{734sample})/OD_{734control}] *100$

Reducing power activity. The reducing power assay was performed using the method described by Gunasekaran *et al.* (2017). Fungal extract was mixed with 2.5 mL 0.2 M phosphate buffer (pH 6.6) and 2.5 mL 1% potassium ferricyanide. The mixture was incubated for 20 min at 50°C. After incubation, 2.5 mL 10% trichloro acetic acid was added to the mixture and then centrifugation was done at 3000 rpm for 10 min. Subsequently, 2.5 mL of the supernatant was taken in separate test tube to which 2.5 mL distilled water and 0.5 mL 0.1% ferric chloride was added. The absorbance of the resulting solution was read at 700 nm against a blank. Ascorbic acid was used as a standard.

Determination of fungal trehalose content. An enzymatic assay as described by Al-Bader *et al*. (2010) was used to determine the trehalose content of the fungal endophytes. Endophytic fungi were grown on PDA agar for 7 days at 30ºC. One disc (5 mm) of each fungal culture was inoculated in 100 mL of PDA broth and PEG amended PDB, then incubated at 30ºC for 14 days. After 14 days, mycelium was separated by filtration using sterile filter paper. Mycelium collected were immediately lyophilized using liquid nitrogen. Lyophilized samples were ground in prechilled pestle and mortar. Trehalose was extracted from known weight of lyophilized mycelium powder in water by heating at 95 to 98°C for 3.5 h. Samples were then treated with 1 M acetic acid (75µL) and 0.2 M sodium acetate (300 µL). The mixture was centrifuged at 1000rpm for 5minutes. Supernatants were collected and treated with 0.05 U/ mL of trehalase (3 µL) from porcine kidney and the other set left untreated was a control. Reaction mixture was mixed well and incubated overnight at 37°C for the conversion of trehalose to glucose. The glucose concentration in all samples was assayed by the glucose oxidase method described by Huggett and Nixoh (1957) with slight modification. The glucose oxidase peroxidase reagent was freshly prepared by dissolving 25mg of glucose oxidase and 1% ortho- toluidine in sodium phosphate buffer (0.05M, pH 7.2). To the mixture small quantity of peroxidase (2mg) was added and volume made up to 25 mL using buffer. The test samples (50 µL) were treated with glucose oxidase peroxidase reagent (100 µL) and incubated at 37°C for 30 minutes. Later, 9N H_2SO_4 (100 µL) was carefully added to the mixture to stop the reaction. The absorbance was read at 540 nm using a spectrophotometer (UV-VIS, Systronics Ltd., India. The glucose concentration was calculated based on a calibration curve using glucose as the standard. The trehalose content of the samples was determined by subtracting glucose concentration of the sample with the glucose concentration of the control.

RESULTS AND DISCUSSION

Evaluating plant growth promoting activity of fungal endophytes. All the six fungal endophytes formed halo zone around the colony when

grown on Pikovskaya's Agar medium (Fig. 1, 2). This suggested that the fungal endophytes were able to solubilize tri-calcium phosphate in the medium by the production of organic acids or inorganic acids (Khan *et al.*, 2014). Isolate P10R1 showed highest phosphate solubilization index compared to other endophytes (Fig. 1). Similarly, Adhikari and Pandey (2019) demonstrated the potential of endophytic fungi (*Penicillium* and *Aspergillus* spp.) isolated from roots of *Taxus wallichiana*, for their ability to solubilize insoluble phosphates through production of phosphatases, phytases and organic acids. None of the fungal endophytes showed the ability to solubilize potassium in Aleksandrow agar medium. Isolates P10R1 and P12R2 produced siderophore forming halo zone around the colony when grown on CAS agar medium (Fig. 2b, Fig. 1). Siderophores are the iron-chelating agents that stimulate plant growth by facilitating insoluble iron acquisition to the plants. Kajula *et al.*, 2010 reported that the production of extracellular siderophore by the foliar endophytic fungi isolated from Scots pine (*Pinus sylvestris* L.) and labrador tea.

Phytohormones are essential for plant growth and development, as well as plant interactions with microorganisms. Microbes also have the ability to biosynthesis phytohormones as secondary metabolites. Indole acetic acid (IAA) produced by fungal endophytes ranged between 0.13-0.6 mg/ mL, which was reduced to 0.08-0.28 mg/ mL when they were grown in matric modified medium (Fig 3a). Isolate P10R1 (0.28 mg/ mL) and P6R4 (0.6 mg/ mL) showed highest production of IAA under stress and normal condition, respectively. Fungal endophytes also produced GA in significant quantity where isolates P1R1 (0.13 mg/ mL) and P6R4 (0.094 mg/ mL) showed highest GA production under normal and stress condition respectively (Fig. 3b). Exogenous production of IAA and GA both under stress and normal conditions suggested that these fungal endophytes are capable of inducing growth promotion in host plant. This finding is similar with the study conducted by Khan *et al.* (2012) who reported that the secretion of phytohormones IAA and GA by the fungal endophytes *Paecilomyces formosus* LHL10 promoted the host plant growth and alleviated adverse effects of salt stress. Salicylic acid (SA) produced by fungal endophytes ranged between 0.019-0.167 mg/ mL under normal condition and 0.003-0.121 mg/ mL under stress condition (Fig. 3c). Isolate P10R1 (0.167 mg/ mL) and P1R1 (0.121 mg/ mL) showed significant increase in the production of SA under normal as well as stress conditions respectively. Fungal endophytes also produced ABA in a significant amount where isolate P1R1 showed highest production of ABA under both stress and normal conditions (Fig. 3d). ABA acts as a signaling mediator in plants to regulate plants response to environmental stresses (Sah *et al.*, 2016). The findings are in agreement with the report of Javed *et al.* (2022) who reported increased production of IAA, GA, ABA and SA by the fungal endophytes under PEG induced drought stress.

Evaluating stress alleviating traits of fungal endophytes

Fungal trehalose content. Trehalose is a nonreducing disaccharide of glucose that is synthesized by fungi, which functions both as a reserve carbohydrate and as a stress protectant. Trehalose accumulation help in the osmotic adjustments of the cell under water stress condition. In this study significant increase in trehalose content was observed in mycelia of all 6 isolates when grown under PEG induced stress condition (Fig. 4a). The Isolate P10R1 (12.21 µg/ mg) produced highest trehalose followed by P6R4 (11.89 µg/ mg) and P1R1 (9.76µg/ mg) under stress condition. This suggests that the fungal endophytes accumulate trehalose to overcome the water stress condition. The Ramos *et al.* (1999) reported trehalose as a predominant sugar, accumulated in conidia and mycelia of *Aspergillus ochraceus* grown in matric modified media. Ramirez *et al.* (2004) reported increase in the content of trehalose in *Fusarium graminearum* under osmotic and matric potential stress.

Total phenols and flavonoids. Phenols and flavonoids are the antioxidant compounds produced by plants and fungi. An increase in phenol and flavonoid content in culture filtrate of all the isolate was observed under stress condition compared to normal condition (Fig. 4b and 4c). Highest phenol and flavonoid content were observed in culture filtrate of isolate P6R4 (1.41 mg/ mL) and P10R1 (52.98 µg/ mL) respectively when grown in matric modified media. Javed *et al.* (2022) reported increased production of phenol and flavonoids by the fungal endophytes (WA, TG and TL3) under PEG induced stress.

ABTS $^{+}$ scavenging activity and Reducing power activity. Antioxidant activity of the fungal extract was evaluated based on the ability to scavenge synthetic radicals ABTS $^{+}$ (Fig. 5a). An increase in antioxidant activity was observed in fungal extract of all isolates when grown in matric modified media. Isolate P10R1 (94.13%) showed maximum antioxidant activity under stress condition followed by P6R4 (88.81%). Reducing power assay represents the antioxidant activity of the compound that breaks the free radical chain through donation of a hydrogen atom (Ravindran *et al.*, 2012). An increase in reducing power activity was observed in all the isolates under stress condition (Fig. 5b). However, isolate P10R1 showed highest reducing power activity both under stress and normal condition. The results revealed that the endophytic fungi are capable of producing antioxidant compounds and protect themselves as well as their hosts from oxidative stress. Hameed *et al.* (2017) reported a significant antioxidant and reducing power activity of the filamentous fungi *Mucor circinelloides.*

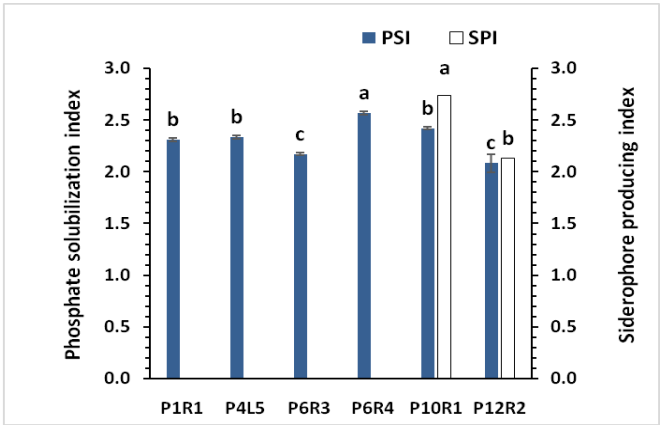

Fig. 1. Phosphate solubilization index and siderophore producing index of fungal endophytes. Means ± SEM are shown. Bars with same alphabets do not differ significantly at p≤0.05 as per Duncan Multiple Range Test (DMRT).

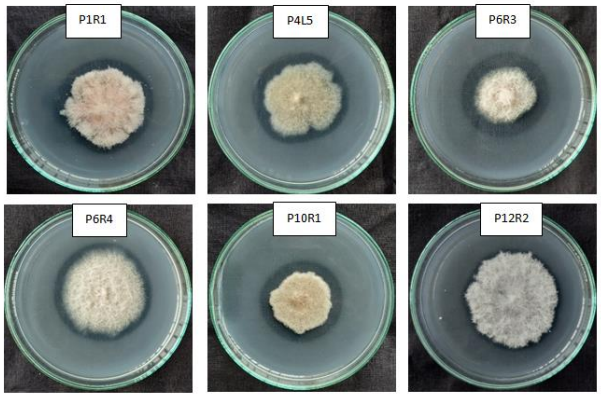

Fig. 2a. Phosphate solubilization activity of fungal endophytes on Pikovskaya medium.

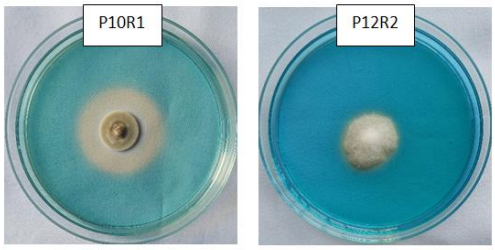

Fig. 2b. Siderophore production by fungal endophyte on CAS agar medium.

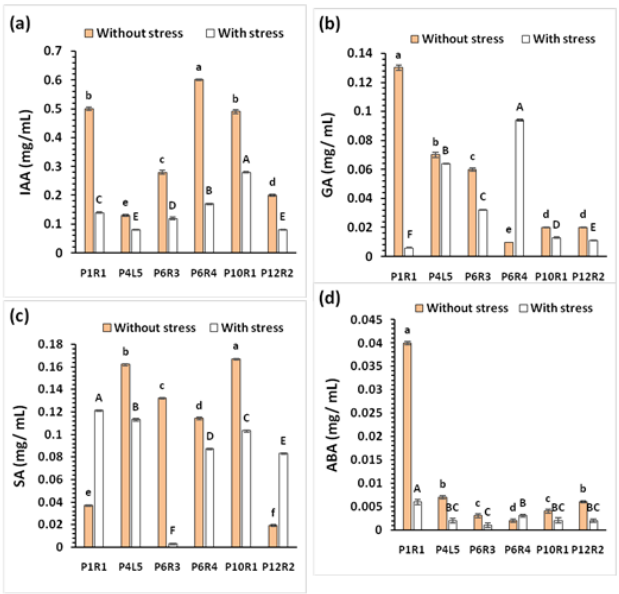

Fig. 3. Phytohormones secreted by fungal endophytes. **(a)** Indole acetic acid, IAA; **(b)** gibberellic acid, GA_3; **(c)** abscisic acid, ABA; **(d)** salicylic acid, SA. Means ± SEM are shown. Bars with same alphabets do not differ significantly at p≤0.05 as per Duncan Multiple Range Test (DMRT).

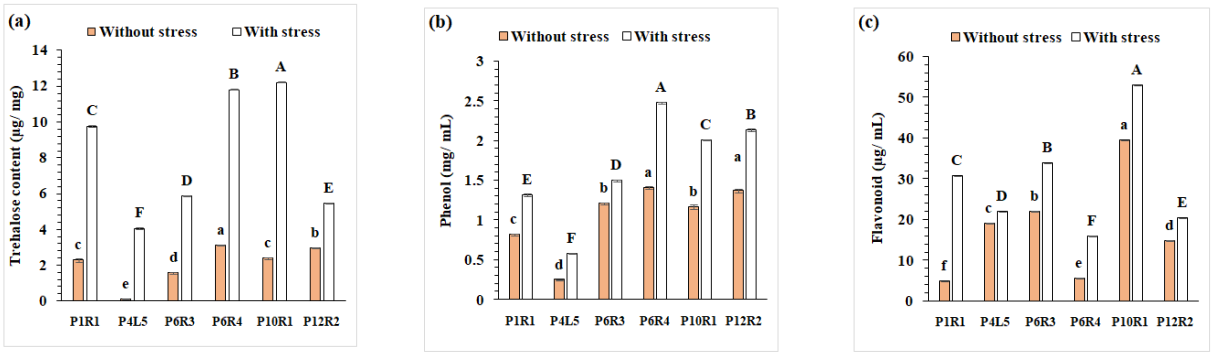

Fig. 4: (a) Trehalose; **(b)** phenols; **(c)** flavonoids secreted by fungal endophytes. Means ± SEM are shown. Bars with same letters do not differ significantly at p≤0.05 as per Duncan Multiple Range Test (DMRT).

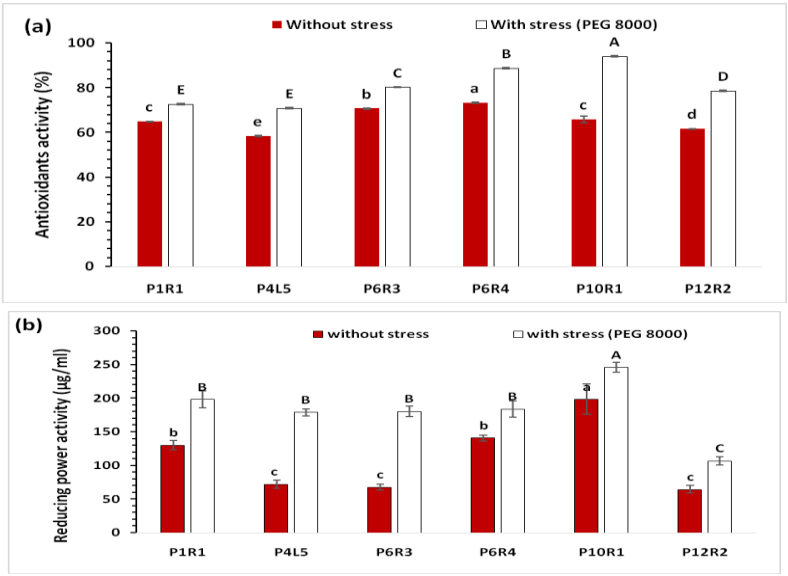

Fig. 5. Antioxidant activity of fungal endophytes (**a)** $ABTS^{+}$ scavenging activity (**b)** Reducing power activity. Means ± SEM are shown. Bars with same letters do not differ significantly at p≤0.05 as per Duncan Multiple Range Test (DMRT).

CONCLUSIONS

Fungal endophytes are widely known to impart abiotic stress tolerance in host plants. In this study, fungal endophytes were able to produce growth hormones both under PEG induced stress and normal condition. Also, they were able to solubilize phosphate and produce siderophores. Further, they showed antioxidant activity and accumulated trehalose content in fungal mycelia under drought stress. Overall, this study gives a comprehensive understanding of the mechanism involved in imparting drought tolerance in host plants by fungal endophytes.

FUTURE SCOPE

This study motivates to advance investigation of selected fungal endophytes in order to develop a bioagent imparting drought tolerance in crops with applicability to multifield.

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Conflict of Interest. None.

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