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Fungal Endophytes Isolated from Drought Adapted Plants Improve Maize (*Zea mays* L.) Seedling Growth under PEG induced Drought Stress

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Abstract

Endophytes are one of the symbiotic associations which can be successfully utilized to mitigate negative effects of abiotic stress in agricultural crops**.** Habitat adapted symbiotic fungal endophytes are known to impart drought tolerance in host plants. This study aimed at evaluating fungal endophytes of drought adapted plants against drought stress in maize. Fungal endophytes were isolated from arid and semi-arid regions of Karnataka and screened for their ability to grow in matric modified media using Polyethylene glycol (PEG 8000). Out of 65 isolates, six isolates (P1R1, P4L5, P6R3, P6R4, P10R1 and P12R2) showed increased mycelial growth up to -10.31 MPa (30 % PEG). The six fungal endophytes were evaluated for their ability to alleviate drought stress and improve growth of maize seedlings at PEG induced drought stress under *in vitro* condition. Maize seedlings inoculated with fungal endophytes showed significant increase in shoot length under drought stress after 10 days compared to control. Among six endophytes, the isolate P10R1 significantly enhanced shoot and root length of maize. The fungus was identified as *Alternaria burnsi* by using ITS region sequences. This study revealed that the fungal endophyte, *Alternaria burnsi* isolated from drought adapted plant has ability to mitigate drought stress and improve the maize growth under drought condition.

Keywords : Fungal endophyte, PEG 8000, Drought, Maize

Fungal endophytes colonize plant tissues in intercellular spaces without causing any disease symptoms. Mutualistic or symbiotic association of plant with endophytic fungi confer fitness benefits to host plant which include tolerance to biotic and abiotic stresses (Sherameti *et al.,* 2008 and Rocha *et al.,* 2011). Among abiotic stresses, drought is one of the important abiotic factors expected to cause reduced growth and yield. It is estimated that more than 50 per cent of arable land may be affected with drought by 2050 (Vinocur and Altman, 2005). Plants response to the drought stress is a complex phenomenon. Plants adapt various metabolic and physiological mechanisms to tolerate or avoid stresses. Plant associated microbial community, particularly the fungal endophytes play a critical role in assisting host plant to overcome abiotic stresses. Fungal endophytes are known to impart drought tolerance in host plant by activating host defense systems such as osmotic adjustment, increasing plant growth by producing growth hormones, scavenging reactive oxygen species (ROS) and producing antioxidant enzymes (Ali *et al.*, 2018; Moghaddam *et al.,* 2021; Khan *et al*., 2016). Rodriguez *et al.* (2009) classified fungal endophytes into 4 classes where clavicipitaceous endophytes were grouped into class I fungal endophytes and non clavicipitaceous endophytes into Class II, III and IV. Fungi colonizing above and below-ground plant tissues and being horizontally and vertically transmitted were grouped as class II fungal endophytes. Class III endophytes were defined to contain mostly members of the *Dikaryomycota* (*Ascomycota* or *Basidiomycota*), which are particularly well studied in trees, but also

in other plant taxa and in various ecosystems. Class IV endophytes comprise dark, septate endophytes, which, similar to mycorrhizal fungi, are restricted to roots, where they reside inter and intracellularly in the cortical cell layers. The Class II endophytes have broad host range and are readily culturable on artificial media and are thought to colonize all plants in natural ecosystems. Many studies have been reported the benefits of habitat adapted endophytes in plants against abiotic stresses which includes drought, heat and salinity (Rodriguez *et al.* 2008; Redman *et al.,* 2002 and Manasa *et al*., 2015). Ghaffari *et al.* (2019) reported *Piriformospora indica* colonization readjusts plant metabolites and sustains the presence of aquaporins in drought-stressed plants. Moghaddam *et al.* (2021) reported fungal endophyte *Periconia macrospinosa* induce drought tolerance in tomato and cucumber by increasing proline content and antioxidant activities in host plant. Therefore, fungal endophytes isolated from drought adapted plants can impart drought stress tolerance in crop plants. This study is intended to screen and evaluate efficient fungal endophytes against drought stress in maize.

Material and Methods

Isolation of Fungal Endophytes

From arid and semi-arid regions *viz.,* Bellary, Koppal, Chinthamani, Chithradurga, twelve grass species were collected which belong to different agro-climatic zones *viz.,* North eastern dry zone, Central dry zone and eastern dry zone of Karnataka (Table 1). Root and leaf samples were cut in to 1cm bits and surface sterilized using 4 per cent sodium hypochlorite for 45 seconds followed by 70 per cent alcohol for one minute. The sterilized bits were repeatedly washed using sterile water to remove residual chemicals. The surface sterilized root and leaf bits were placed on Potato Dextrose Agar (PDA) medium dispensed plates and incubated at 28 °C for 7 days. The fungal mycelia emerged from the medium were purified and preserved under refrigerator for further study.

Screening of Fungal Endophytes for Drought Tolerance

Under matric induced water stress, sixty one fungal isolates were screened. Actively growing mycelial

Table 1

Details of location of plant samples collected and designated endophytic fungal isolates

Place (Agro climatic zones of Karnataka)	Location	Plant sample	Isolate codes
Koppal (Northern dry zone)	Achalapur	*Brachiaria mutica*	P1R1, P1R2, P1R3
	Raghunatahn halli	*Fimbristylis miliacea*	P2R1, P2R2, P2R3, P2R4, P2L1, P2L2, P2L3, P2L4, P2L5
Bellary (Northern dry zone)	Tondehal	*Panicum repens*	P3R1, P3R2, P3R3, P3L1, P3L2, P3L3, P3L4, P3L5
	Desanur	*Digitaria ciliaris*	P4R1, P4R2, P4R3, P4L1, P4L2, P4L3, P4L4, P4L5
Chikkaballapura (Eastern dry zone)	Chinthamani	*Dichanthium* spp	P5R1, P5R2, P5R3, P5R4
	Kurubur	*Eleusine indica*	P6R1, P6R2, P6R3, P6R4, P6R5, P6L1, P6L2
	Shidlaghatta	*Sorghum halepense*	P7R1
Chitradurga (Central dry zone)	Hiryuru	*Tragus* spp.	P8R1, P8R2, P8L1
	Hiryuru	*Digitaria* spp.	P9R1
	Ramajogihalli	*Cyperus* spp.	P10R1, P10R2, P10L1, P10L2
	Ramajogihalli	*Arthraxon* spp.	P11R1, P11R2, P11R3, P11R4, P11R5, P11L1, P11L2, P11L3
	Challakere	*Urochloa* spp.	P12R1, P12R2, P12L1, P12L2, P12L3

Note: P-Plant, R-Root, L-leaf

discs (5mm) were made using sterile cork borer. These discs were inoculated into Erlenmeyer flask containing Potato Dextrose broth amended with Polyethylene glycol (PEG 8000) at 15, 20, 25 and 30 per cent concentrations which corresponds to -2.88, -4.85, -7.33 and -10.31 MPa water potential respectively (Michel, 1983). The inoculated flasks were placed in a shaker (90 rpm) at 25 °C for 7 days. Fungal mycelia were separated by passing the culture broth through pre-weighed Whatman - No.1 filter paper. The filter paper was rinsed in distilled water to remove broth residue. The filter paper with mycelium was dried at 60 °C in hot air oven for 48 hrs to attain constant weight and the final weight of filter paper with mycelium was recorded. The dry weight of the fungal mycelium recorded was the final weight of the filter paper with mycelium minus weight of the filter paper. Fungal growth types were assessed as reported by Hutton *et al*. (1996).

Morphological Characters of Selected Drought Tolerant Fungal Isolates

The fungal growth was observed for their colony morphology and the fruiting body and spore characters were studied under microscope using agar slide culture technique (Harris, 1986).

Evaluation of Selected Fungal Endophytes for Induction of Drought Stress Tolerance in Maize

Maize seeds (MAH 14-5) were surface sterilized using sodium hypochlorite (4%) solution followed by 70 per cent ethanol and repeatedly washed with sterile water to remove residual chemicals on the seeds. Then these seeds were pre-germinated in sterile moist blotters. The pre-germinated seeds were treated with fungal spore suspension (10^6 spores/mL) prepared from 10 days old culture for 3 hours (Zhang *et al*., 2014). The control seeds were treated with sterile distilled water. The pre-germinated seeds were subjected to drought stress by placing them on a sterile paper towel soaked in 14.6 per cent (LC_{50}) PEG (Roopashree, 2022) and incubated at 27 ºC in the growth chamber for 10 days. There were three replications and each replication comprised with fifteen seedlings. Root and shoot lengths were recorded after incubation for 10 days.

Confirmation of Fungal Endophytes in the Tissue of Inoculated Maize Seedlings

The fungal endophytes were re-isolated from the inoculated seedlings on PDA medium and confirmed by comparing their colony morphology and spore characteristics with the mother cultures.

Identification of the Efficient Drought Tolerant Fungal Isolate P10R1

The genomic DNA of the fungal isolate was extracted by CTAB (Cetyl trimethyl ammonium bromide) method (Lee *et al.,* 1988). The fungal mycelia (500 mg) were macerated using sterile pestle and mortar in liquid nitrogen and a pinch of Poly vinyl pyrolidone (PVP) to obtain a fine powder. The powder was added to 3mL extraction buffer (50 mM Tris-HCl of pH 8.0, 50 mM EDTA, 0.7 M NaCl, pinch of PVP and 2 µl of β-mercaptoethanol), mixed gently and incubated at 65 ºC for 45 min with intermittent shaking. The lysate was extracted by adding an equal volume of chloroform: isoamyl alcohol (24:1) and centrifuging at 10,000 rpm for 10 min. The supernatant was transferred to new tube and further chloroform: isoamyl alcohol (24:1) was added and centrifuged again. This step was repeated, until the middle layer disappeared. The genomic DNA was precipitated by adding chilled isopropanol (600 µL) and incubated at -20°C overnight. After incubation, the tubes were centrifuged at 10,000 rpm for 10 min and the aqueous layer was discarded and the pellet was washed by using 500 µl of 70 per cent chilled ethanol. The pellet so obtained was air dried and dissolved in 20 µl of deionized sterile water. The dissolved pellet was treated with five µl RNase A enzyme and incubated at 37°C for one hour. The DNA was subjected to electrophoresis using 0.8 per cent agarose gel, gel documented and purity was checked using Nano drop. This DNA was used as template for amplification.

Polymerase Chain Reaction (PCR)

The internal transcribed spacer (ITS) region of genomic DNA was amplified using universal primer ITS1-F (5[TCCGTAGGTGAACCTGCGG 3[) and ITS4-R (5[TCCTCCGCTTATTGATATGC 3]) by polymerase chain reaction (PCR). PCR amplification was performed using thermocycler (Mater cycler Nexus gradient, Eppendrof, India) with a 20 µL reaction mixture that comprised of 2 µL 1X taq buffer with $MgCl_2$ (1.5 mM), 2µL dNTP's (10mM), 0.5 µL each primer (10 pmol), 0.3 µL Taq DNA polymerase (3U) and 1µL template DNA (100 ng). The PCR was carried out with an initial denaturation at 96 °C for 4 min, followed by 35 cycles at 94 °C for 30s, 55 °C for 1 min and 72 °C for 30s and a final extension at 72 °C for 12 min. Then the amplified product of DNA was electrophoresed using one per cent agarose gel. The DNA band was visualized under UV light and documented using a gel documentation unit (Vilber, E-Box CX5.TS, France). The amplified DNA was eluted by using gel extraction kit to obtain purified PCR product. The eluted DNA was sequenced by Barcode bioscience pvt. Ltd., Bangalore, India. The sequence data received from the company was analysed for homology using NCBI GenBank.

Sequence Analysis and Homology Search

Sequence results were analysed using the online software National Centre for Biotechnology Information (NCBI), USA. The BLAST (Basic Local Alignment Search Tool) search was done for partial length sequence homology with NCBI data (http// www. Ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1990). Phylogenetic analysis was done by using MEGA10 software and a phylogenetic tree was generated using neighbour-joining algorithm.

Statistical Analysis

The data was statistically analysed using WASP: 2.0 (Web Agri Stat Package 2) statistical tool (www.icargoa.res.in/wasp2/index.php) and the means were separated by Duncan Multiple Range Test (DMRT).

Results and Discussion

Screening of Fungal Endophytes Drought Tolerance

Sixty one endophytes were isolated from 12 plant species of arid and semi-arid regions of Karnataka and screened for drought tolerance (Fig. 1 & Table 1) using different concentrations of PEG. The isolates grown in PEG amended medium showed 3 different types (Type-I, Type-II and Type-III) of growth response as defined by Hutton *et al*. (1996) which include: Type I- overall minimal growth after 7 days; Type II- maximum growth at control with decreased growth as matric water stress increased; Type III- maximum growth under some degree of matric induced water stress. Out of sixty-one isolates (Fig.1 & Fig.2), 38 isolates showed decreased mycelial dry weight with increased PEG concentration indicating that they possess type-II growth response. Seventeen isolates showed no

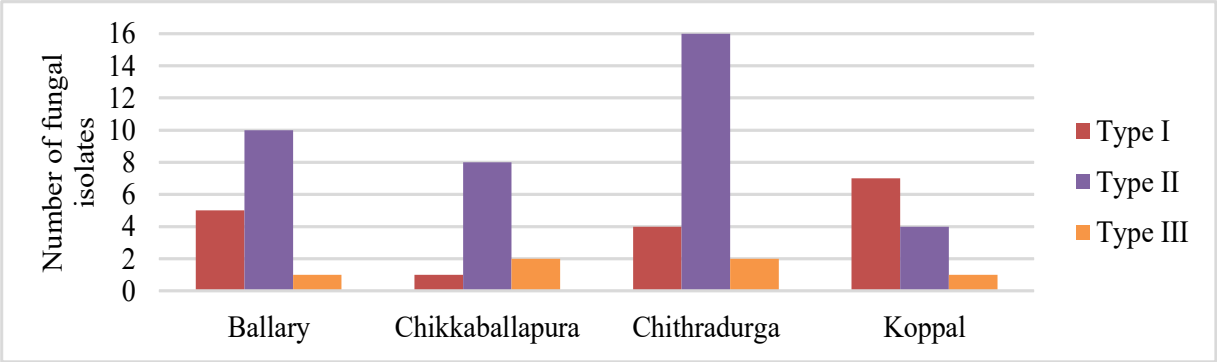

Fig. 1: Number of fungal endophytes isolated from drought adapted plants from various parts of Karnataka showing different growth type response

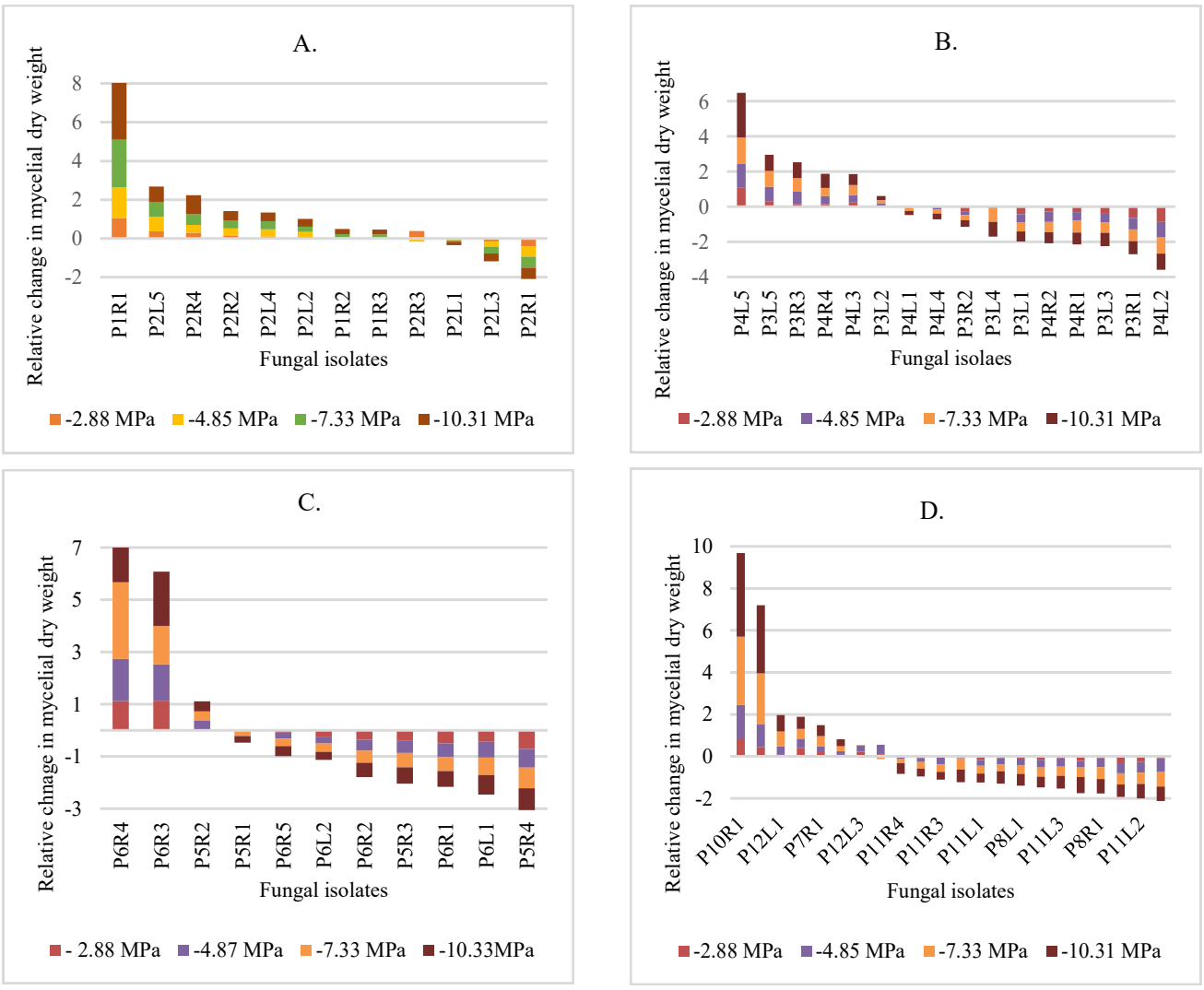

Fig. 2: Relative change in mycelial dry weight of fungal endophytes with respect to control (mycelial dry weight of isolates grown in PD broth) at different water potential
Locations: A) Bellary B) Chikkaballapura C) Chitradurga D) Koppal districts of Karnataka

response to increased PEG concentration (decreased water potential) indicating that they are belong to type I growth response. Remaining 6 fungal endophytes (P1R1, P4L5, P6R3, P6R4, P10R1 and P12R2) showed increased mycelial dry weight with increased concentration of PEG (-10.31MPa) compared to control and belong to the Type III indicating that they are more tolerant to matric-induced water stress (Ramirez *et al.,* 2004). The preference of these six endophytes for low water potentials in the study might be due to their arid habitats and the low water potential of host plants in arid regions (Moghaddam *et al.,* 2021). Osmotic potential and matric potential could be considered two important components of water potential which influence the water availability for the microorganisms (Aujla and Paulitz, 2017). Osmotic potential is contributed by the solutes in the water and matric potential by the interaction of water with solid phase (Papendick and Campbell, 1981). Most fungi tolerate osmotic-induced water stress better than matric-induced water stress (Ramirez *et al.,* 2004). This suggests that the matric induced water stress tolerance would be an important factor that could be considered to screen drought tolerant fungal endophytes. Polyethylene glycol (PEG) is a high

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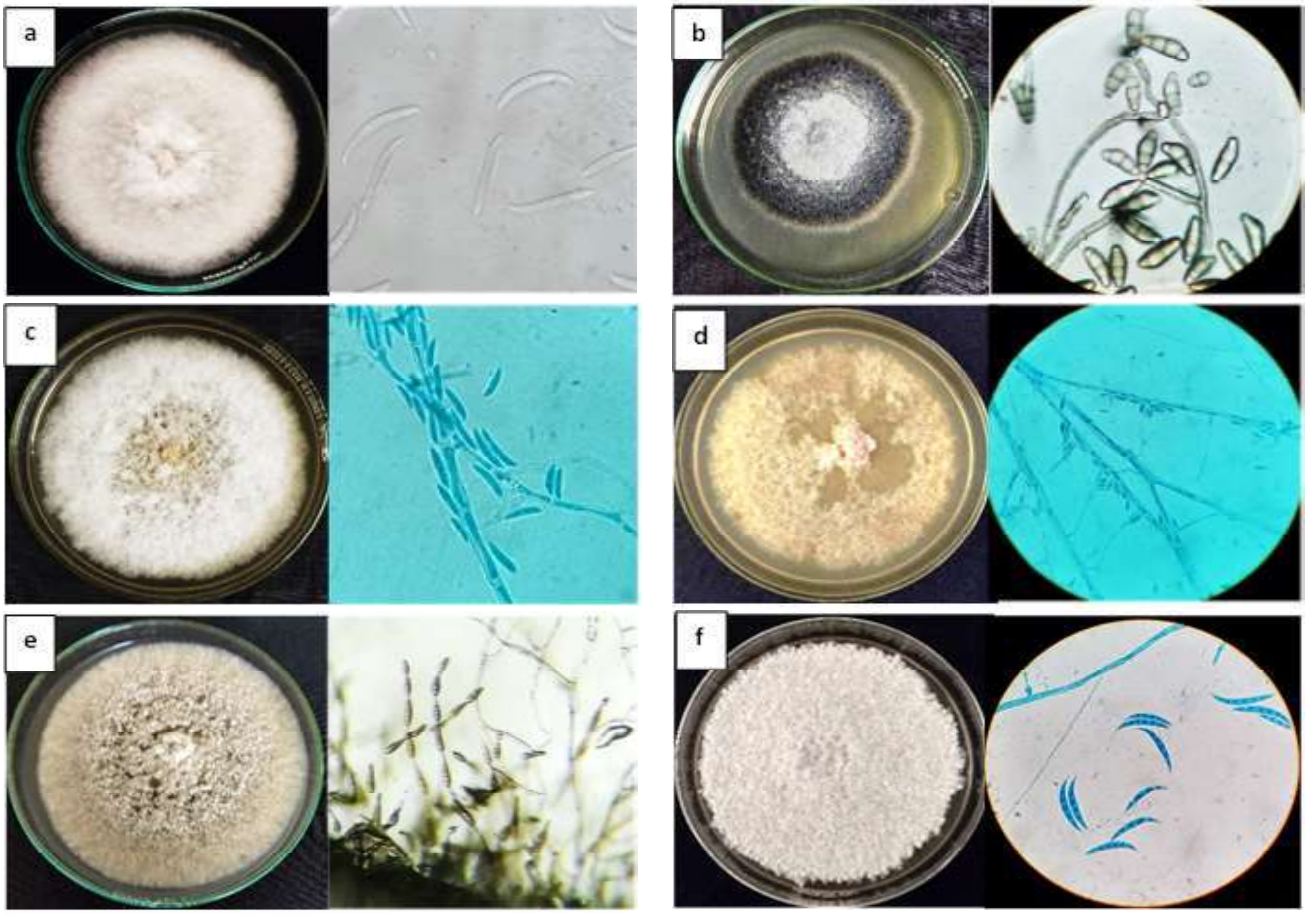

Plate 1: Mycelial growth of endophytic fungi on PDA and spore structures under microscope (40x) a. *Fusarium* P1R1, b. *Curvularia* P4L5, c. *Fusarium* P6R3, d. *Fusarium* P6R4, e. *Alternaria* P10R1, f. *Fusarium* P12R2

molecular weight compound which is impermeable through both cell wall and cell membrane of microorganisms and contributes to the matric induced water stress (Harris, 1981). Hence, growth media was amended with polyethylene glycol (PEG) with different concentration which corresponds to various matric induced water potential. Roopashree and Rajendra Prasad (2021) demonstrated the Type III growth response (-7.41 MPa PEG) in the fungal endophyte isolated form the plants grown in extreme climatic conditions. The six selected fungal endophytes (P1R1, P4L5, P6R3, P6R4, P10R1 and P12R2) when examined for their cultural, microscopic and morphological characteristics (Plate1, a,b,c,d,e and f) revealed that four isolates P1R1, P6R3, P6R4 and P12R2 belong to the genera of *Fusarium*, P10R1 to *Alternaria* and P4L5 to *Curvularia* (Watanabe, 2010).

In vitro Evaluation of Selected Fungal Endophytes on Growth of Maize under PEG Induced Drought

The six isolates (Fig. 2) having maximum mycelial dry weight at -10.31MPa water potential were further evaluated for their ability to influence on early seedling growth of maize (MAH 14-5). The seedlings inoculated with fungal endophytes showed significant increase in shoot length compared to un-inoculated seedlings under stress. The fungal endophytes did not influence on the root length of maize seedlings which are not treated with PEG. But, significant increase in root length was observed in maize seedlings grown under PEG induced drought conditions (Table 2). In addition, root hair development was observed in endophyte inoculated seedlings. This indicates that the fungal endophytes might have influenced

TABLE 2

Influence of fungal endophytes on shoot and root length of maize under without and with drought stress

Treatments	Without drought stress		Drought stress (-2.74 MPa)	
	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)
Control	13.70 ^{e}	23.20	5.60 ^{e}	15.10 ^{e}
P1R1	17.60 ^{b}	20.80	7.90 ^{d}	16.50 ^{cde}
P4L5	15.24 ^{d}	23.50	8.24 ^{cd}	17.70 ^{cd}
P6R3	16 ^{cd}	24.26	8.90 ^{bcd}	16.10 ^{de}
P6R4	18.98 ^{a}	24.86	10.18 ^{b}	21.10 ^{b}
P10R1	19.04 ^{a}	24.90	12.36 ^{a}	23.02 ^{a}
P12R2	17.30 ^{bc}	24.40	9.90 ^{bc}	18.26 ^{c}
C.D (0.05)	1.32	NS	1.75	1.77
C.D (0.01)	1.79	NS	2.36	2.39

Note: Means of same superscript in a column do not differ significantly at p=0.05 as per DMRT; NS= Non significant

root architecture by producing plant growth hormones (Verma *et al.,* 2018). Among the six selected fungal endophytes, the isolate $P_{10}R_1$ showed the highest shoot and root length compared to others. Li *et al*. (2019) reported the increased root length of maize inoculated with fungal isolate (*Paraphoma* sp., *Embellisia chlamydospore* and *Cladosporium oxysporum*) under moderate drought stress. The fungal endophytes were re-isolated from the inoculated seedlings and confirmed by comparing with respective mother cultures.

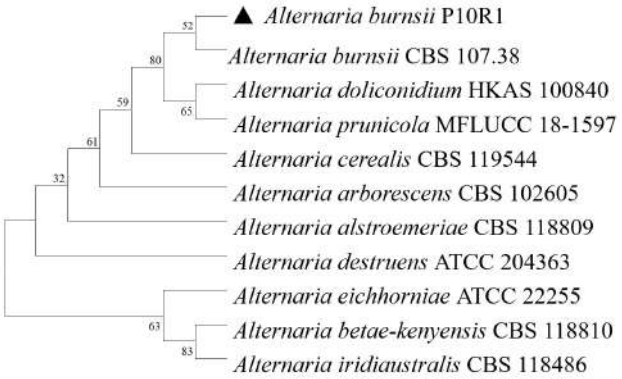

Fig. 3: Phylogenetic tree of *Alternaria* species

Identification of Efficient Drought Tolerant Fungal Isolate $P_{10}R_1$

Ribosomal DNA Internal Transcribed Spacer (ITS) sequence analysis is widely used for the identification of microorganisms. The use of ITS region sequences was proved to be a valuable source of evidence to resolve phylogenetic relationships at lower levels, such as among genera or species. The ITS region sequences of selected fungal isolates were compared with already published sequences in the NCBI data base and fungal endophytes were identified. The ITS partial gene sequence of the $P_{10}R_1$ isolate having the sequence length 505 base pair (bp) showed 99.80 per cent homology with *Alternaria burnsii* available in the NCBI database. The phylogenetic tree (Fig. 3) constructed using the sequences of 10 *Alternaria* species available in the NCBI GenBank showed that the isolate $P_{10}R_1$ is closely related to *Alternaria burnsii* CBS 107.38. Thus, the fungus $P_{10}R_1$ was identified as *Alternaria burnsii*. The ITS region has the highest probability of successful identific ation for the broadest range of fungi with the most clearly defined gap between inter and intra specific variation (Schoch *et al*., 2012), the above said fungal endophyte was identified using sequence homology. It can be concluded from the study that *Alternaria burnsii* as the efficient drought tolerant fungal endophyte which has the ability to impart drought tolerance in maize.

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