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Fungal endophytes of Himalayan Cold Desert Induces Heat tolerance in Rice (*Oryza sativa* L.)

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**Abstract**

The plants growing in cold desert of western Himalaya have inhabited diversified endophytes. These endophytes can provide fitness to plant under harsh environmental situations. In the current study, 22 fungal endophytes isolated from Artemisia and Xerophytic plants growing in the cold desert were screened for thermostolerance at different temperature ranges (28, 30, 32, 34, 36, 38 and 40 °C) under *in vitro*. The only three isolates viz., A2, A7 and X5 exhibited growth up to 40 °C and identified as *Penicillium funiculosum* (A2), *Ceriporia lacerate* (A7) and *Endomelanconiopsis endophytica* (X5) using ITS region. These endophytes inoculated to rice seedlings and exposed to elevated temperature (45 °C) for 7 hr per day for 10 days to study their effect on tolerance of rice to heat stress. The results revealed that endophytes inoculated seedlings showed sustained improvement in shoot and root growth. In addition, endophytes significantly increased the photosynthetic efficiency by regulating photosynthetic rate, transpiration rate and stomatal conductance as well as increased photosynthetic pigments compared to uninoculated seedlings. The *E. endophytica* was chosen to be the best endophyte to impart heat stress as per Fernandez model. This study suggested that cold desert endophytes could induce heat tolerance in plants.

**Keywords:** Cold desert, fungal endophytes, Rice and Heat stress

**Introduction**

The global temperature increases day by day due to change in climate. Frequent heat waves have had serious impacts on rice production (Zhang and White 2021). Historical data analysis envisaged that 7–8% of rice yield has been decreased due to raise in temperature to 1°C (Baker et al. 1992). International Rice Research Institute (IRRI) demonstrated that the field trials from 1992-2003 showed 10% yield reduction of rice for every raise in one degree of minimum temperature (Peng et al. 2004). High temperature affects all stages of rice plant starting from germination, growth, development, reproduction and yield (Krishnan et al. 2011). The tiller number decreased by 10% when temperature rise from 29/21°C to 37/29°C (Manalo et al. 1994). The synchronism between the emergence of main stem and tiller and also mobilization of nutrients among tillers were affected by high temperature resulting in decreased yield as primary tillers are directly proportional to grain yield in rice (Yoshida 1981). Heat stress can cause physiological, biochemical and molecular changes in plants such as increase in membrane fluidity, protein denaturation, level of reactive oxygen species, alteration in leaf gas exchange parameter, inactivation of mitochondrial and chloroplast enzyme activities as well as decline in photosystem II mediated electron transport and boosts up transpiration rate which produces physiological drought that causes reduction in crop biomass and yield (Gururani et al. 2015).
Cold deserts are found in high, flat areas, called plateaus, or mountainous areas in temperate regions of the world. Cold deserts have hot summers but extremely cold winters. The Western Himalayan cold deserts have extremes of hot and cold climate combined with excessive dryness. Soil has light grey, poor in fertility and less water holding capacity. Therefore, these desert plants develop some physiological mechanisms like CAM (Crassulacean acid metabolism), modified leaf and also take the advantage of microbial endophytes to survive in hostile environment (Zhang and White 2021).

The endophytes can colonize the plant tissue without causing any apparent harm and provide fitness under hostile environment. Endophytes can be cultured in-vitro and transfer to compatible secondary plants to obtain similar benefits (Baldani et al. 2000; Redman et al. 2002 and Wang et al. 2021). Endophytes isolated from cold deserts seems to adapt wider range of temperature as cold desert has influenced by fluctuated temperature ranges from -45°C in winter to 40°C in summer (Tewari and Kapoor 2013). Therefore, we used in our study the endophytes isolated from the cold desert plants to understand induction of thermotolerance temperature sensitive rice variety IR-64.

**Materials and methods**

**Screening for thermotolerance of endophytic isolates**

The fungal endophytes isolated from Artemisia and xerophytic plants of Western Himalayan cold desert and preserved at School of Ecology and Conservation Laboratory, University of Agricultural Sciences, Bangalore-560065. The 22 isolates were procured and rejuvenated on potato dextrose agar (PDA) for the present study. The endophytic isolates were screened for temperature tolerance. Isolates were cultured in PDA plates and incubated at different temperature (28 °C, 30 °C, 32 °C, 34 °C, 36 °C, 38 °C and 40 °C) for five days. Fungal growth was measured by radial diameter of colony on fifth day of incubation.

**Molecular identification of thermotolerant endophytic isolates**

The endophytic isolates of genomic DNA were extracted by Cetyltrimethylammonium bromide (CTAB) method (Vainio et al. 1998). The internal transcribed spacer (ITS) region of genomic DNA was amplified using universal primer ITS1-F (5’ GCCGTAGGTGAACCTGCGG 3’) and ITS4-R (5’ TCCTCCGCTTATTGATATGC 3’) by polymerase chain reaction (PCR). PCR amplification was performed using Master cycler (Eppendorf, Germany) with a 20µl reaction mixture that comprised 2µl 1X taq buffer with MgCl₂ (1.5mM), 2µl dNTP’s (10mM), 0.5 µl each primer (10pmol), 0.3µl Taq DNA polymerase (3U) and 1µl template DNA (100ng). The PCR was carried out with an initial denaturation at 94 °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. The PCR amplified products were sequenced by SciGenome labs, Cochin, Kerala, India. The nucleotide sequences were queried in the NCBI GenBank database using a Basic Local Alignment Search Tool (BLAST). Sequences of each fungal species and corresponding reference sequences from GenBank were subjected to ClustalW analysis. The phylogenetic tree was constructed through maximum likelihood method and Tamura-Nei model, using MEGA X. The recognized sequences were placed in GenBank with accession number.

**Interaction of fungal endophytes with Rice under heat stress**
Evaluation of fungal endophytes on their ability to impart heat tolerance in rice (variety IR-64) was carried out in plant growth chamber at Indian Institute of Horticulture Research (ICAR-IIHR), Hesaraghatta, Bangalore. There were two sets of experiments. 1. Heat stress (45 °C for 7h per day for 10 days) and 2. Without heat stress (normal temperature conditions, 30±0.5 °C). Each set comprised with following treatments. 1. Control (uninoculated plants) 2. Ceriporia lacerate 3. Endomelanconiopsis endophytica and 4. Penicillium funiculosum. Rice seeds were surface sterilized using 3 % sodium hypochlorite followed by 70 % alcohol. The surface sterilized seeds were repeatedly washed with sterile water and soaked for overnight. The pre-germinated seeds were sown in pots filled with soil and FYM (1:1w/w). Three seedlings per pot were maintained and grown for fifteen days. The thermotolerant endophytes were inoculated by stem prick method (Bhunjun et al. 2020) and allowed to colonize for 10 days. After colonization, set-1 seedlings were exposed to heat (45 °C) for 10 days in growth chamber. Observations for plant height, number of tillers, number of leaves, root volume, fresh and dry weight of roots were recorded after 10 days of heat exposure. Similarly, observations for plants grown under normal conditions (set-2) were recorded. Photosynthetic parameters such as net photosynthesis rate, transpiration rate, stomatal conductivity and intercellular CO₂ concentration were recorded on 10th day of heat stress using a portable gas exchange analyser (LI-6400-XT, LI-COR, NE, USA). The chlorophyll content was estimated by DMSO method (Arnon 1949). Melondialdehyde (MDA) content was determined by tribarbitaric acid (TBA) method (Hodges et al. 1999). Leaf relative water content (RWC) was also determined (Barrs and Weatherley 1962) and calculated using the formula: RWC = (FW − DW)/(TW − DW) × 100, where FW is leaf fresh weight, DW is leaf dry weight and TW is turgid weight.

**Statistical Analysis**

The data generated during experimentation was analyzed by one-way analysis of variance and means were separated by Duncan’s Multiple Range Test (DMRT) using the software XL STAT. The 3-D plot of stress tolerance index (STI) of biomass was constructed according to Fernandez (1992) model using iPASTIC online tool kit (https://manzik.com/ipastic/).

**Results**

**Screening and identification of thermotolerant fungal endophytes**

All endophytic isolates showed good growth up to 30 °C, beyond that there is gradual reduction in growth. This indicated that the optimum temperature of these isolates ranges from 28 to 30 °C. Three isolates viz., A2, A7 and X5 recorded tolerance level up to 40 °C (Table 1). Hence, these isolates were selected for identification and further experiment.

Thermotolerant isolates were identified using ITS regions of rDNA and BLAST search. All the isolates showed 98% similarity with Penicillium funiculosum strain C2-20, Ceriporia lacerate strain BHU MS1 and Endomelanconiopsis endophytica strain CR3 respectively (Fig. S1). The isolates A2, A7 and X5 belongs to three different genera, namely Penicillium funiculosum, Ceriporia lacerate and Endomelanconiopsis endophytica (Fig.1) and the obtained sequences were deposited in GenBank under the accession no. OM368442, MT899187 and MT900590 respectively. The molecular identification was reconfirmed by their macro- and micro-morphological characteristics (Fig. S2). The colony of P. funiculosum was greyish green with funiculose texture on PDA media and examined biverticillate conidiophore with subterminal branches and ellipsoidal
conidia. In case of C. lacerate, white fluffy colonies was observed with aseptate hyphae. Initially colourless colony was observed in E. endophytica and later it become hyaline with shine black color and examined pycnidial conidiomata with ellipsoidal conidia.

**Effects of endophytes isolated from cold desert on imparting thermotolerance in rice**

The fungal endophytes inoculation significantly ($P < 0.01$) improved all growth attributes of rice plants except plant height under both heat stress as well as normal conditions (Table 2 and 3). An endophyte P. funiculosum colonized plants found superior in increasing plant height, number of tillers and leaves, root volume, fresh and dry weight of shoot and root in normal growth condition. Whereas under stress condition, the P. funiculosum and E. endophytica colonized plants showed significantly ($P < 0.01$) higher shoot and root growth parameter compared to C. lacerate. The un-inoculated plants produced least growth of rice.

In normal growth condition, the photosynthetic rate, transpiration and stomatal conductance were significantly increased in endophytes inoculated plants and there were no differences in photosynthetic pigments such as chlorophyll b and carotenoid (Fig. 2, 3 and 4). The reduction of photosynthetic efficiency and pigments were observed in stress induced plants when compared to normal grown plants. However, endophytes inoculation influenced significantly higher ($P < 0.01$) photosynthetic rate compared to uninoculated plants. The E. endophytica inoculated plants showed significantly higher transpiration rate (1.73 mmol m$^{-2}$ s$^{-1}$), stomatal conductance (0.05 mol m$^{-2}$ s$^{-1}$), relative water content (92%) and least MDA content (5.82 mg/g fw.) when compared to other plants (Fig. 2 &3). The total chlorophyll and carotenoid were significantly higher in E. endophytica and P. funiculosum inoculated plants than other plants and these two endophytes had less effect on chlorophyll a:b (Fig. 4).

**Categories of treatments based on their performance in normal and stress conditions**

The treatments were divided into four categories based on Fernandez (1992) model using stress tolerance index of biomass. The treatment E. endophytica inoculated plants belongs to group A that indicates the production of higher biomass under the both conditions (normal and stress). The P. funiculosum and C. lacerate fall under group B having maximum biomass only under normal growth condition. The uninoculated plants formed group D produced least biomass under both the conditions (Fig. 5).

**Discussion**

The numerous studies have been conducted on improvement of crop growth under heat stress using thermotolerant endophytes isolated from harsh environment or wild plants. However, the use of cold desert thermotolerant endophytes were less explored therefore we have analysed the effect of cold desert endophytes on improvement of fitness of rice under heat stress. In present study, the isolates A2, A7 and X5 were observed to be heat tolerant and grown at the range from 28°C to 40 °C. This envisaged that these three isolates could sustain heat stress it might be the cold desert of Western Himalaya had extreme of hot climate (40°C) during summer (Tewari and Kapoor 2013). These endophytes were identified using ITS region of rDNA as P. funiculosum, C. lacerate and E. endophytica. Manasa et al. (2020) identified the fungal OTUs by amplifying the ITS region of the genomic DNA using ITS1 and ITS4 as forward and reverse primers respectively.
High temperature is one of the most important environmental stresses which severely affect the rice growth by reducing the emergence of leaves and tillers resulting in decreased biomass. In the present study, significant higher tiller number was recorded when the plants inoculated with *E. endophytica*, which might positively influenced the new tillers under heat stress by reducing the effects of heat stress on tiller bud. This is in accordance with Vila-Aiub et al. (2005) who reported that *Neotyphodium* sp. infected rye grass produced more tillers than uninfected plants. The endophyte *P. funiculosum* inoculated plants showed highest number of leaves compared to other endophytes which resulted in increased fresh weight of shoot. The root system plays a vital role in adaptation of whole plant under heat stress (Huang et al. 2012). Significant improved in root growth was observed in endophytes colonized plants which lead to improved absorption of nutrients and water from soil, resulting in a more vigorous plant and helps to cope of heat stress. *E. endophytica* was again found better in influencing the root growth compared to others. Our results are in agreement with Waqas et al. (2015) who demonstrated that *Paecilomyces formosus* LWL1 improved root biomass of rice under heat stress.

Heat stress causes the lipid peroxidation of cell membrane which is estimated in terms of MDA (Uemura et al. 2006). Lipid peroxidation indicates oxidative tissue damage, which results in alteration of membrane structure with the release of active peroxy radicals, loss of essential fatty acids and formation of cytosolic aldehyde and peroxide products. The degree of membrane lipid peroxidation depends on MDA level (Ali et al. 2005). Damage to cell membrane indicated by higher concentration of MDA was observed in heat stress exposed plants compared to normal grown plants, however endophytes colonized plants recorded less MDA compared to uninoculated plants under stress. Several studies have also demonstrated that endophytes associated plants had lesser MDA content under abiotic stress (Xu et al. 2017; Ali et al. 2019), which indicates that endophytes involved in cell membrane protection by alleviating the peroxidation of membrane lipids and maintain the fluidity of membrane.

Photosynthesis is one of the most sensitive physiological process to high temperature and is maximum at 30°C in rice (Yamori et al. 2011) and above this temperature assimilation of CO₂ decreases significantly due to inhibition of redox and metabolic reactions, reduction in rubisco activity damage of thylakoid membrane and denaturation of chlorophyll molecules (Mathur et al. 2014; Hussain et al. 2019). In this study, stress exposed plants showed 68.27 % reduction in photosynthetic rate compared to the plants grown at normal conditions due to above factors. These results are in comparison with those of Sonjaroon et al. (2018) who reported 57% reduction in photosynthetic rate when rice seedlings exposed to 40 °C for 7days. However, endophytes colonized plants improved the photosynthetic efficiency by regulating CO₂ assimilation, transpiration rate and stomatal conductance. The results implied that *E. endophytic* colonization could enhance the gas exchange capacity via maintaining of stomatal conductance, which sustains diffusion of CO₂ into the leaves and enhances transpiration cooling (Porch and Hall 2013). Endophytes associated plants have been reported to use significantly less water, increased the biomass than in non-symbiotic plants (Malinowski and Beleskey 2000). This hypothesis was supported by one of our observations, in which the endophytes *C. lacerate* and *P. funiculosum* colonized plants had better water use efficiency by reducing the stomatal conduction and transpiration under stress.

Chlorophyll status is a key index for evaluating plant photosynthetic efficiency. A reduction in photosynthetic rate was linked to the decreases in chlorophyll content due to impaired biosynthesis or
accelerated pigment degradation under heat stress (Camejo et al. 2006). In the present study, the uninoculated rice plants showed 53.49% reduction of total chlorophyll whereas in endophytes colonized plants showed 18.90% reduction. The results revealed that endophytes involved in improving the photosynthetic efficiency by protecting the chlorophyll from denaturation. These results align with those of Xia et al. (2016) who reported that *Epichole* endophyte had significantly higher chlorophyll content and net photosynthetic rate. Carotenoids act as antioxidants and play a crucial role in plant tolerance and adaptation to abiotic stress (Sah et al. 2016). Maximum carotenoid content was observed in endophyte colonized plants than uninoculated plants, which indicates that endophytes could scavenge the reactive oxygen species produced during heat stress. Hence our results revealed that endophytes could improve the biomass of plants by protecting the photosynthetic apparatus. In conclusion, this investigation explored the possibility of using cold desert endophytes for mitigating the heat stress. The endophyte *E. endophytica* seems to be more effective in imparting heat stress tolerance in rice (IR-64).

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References


Figures

Figure 1

Maximum Likelihood tree of the identified fungal endophytes (a) *Penicillium funiculosum* isolate A2) (b) *Ceriporia lacerate* isolate A7 and (c) *Endomelanconiopsis endophytica* isolate X5 and their closest ITS rDNA matches from the GenBank. The phylogenetic tree was constructed with bootstrap value of 500 replicates. Number at the node indicates the bootstrap value.
Figure 2

Influence of fungal endophytes on photosynthetic parameter; (a) Photosynthetic rate (b) Transpiration rate and (c) Stomatal conductance of rice under stress [S] and without stress [WS].

± indicates standard error of mean (n = 4); the dissimilar letters indicate significant difference at P < 0.05 by using Duncan's Multiple Range Test.

C: control (uninoculated plants); Cl: Ceriporia lacerate and En: Endomelanconiopsis endophytica, Pf: Penicillium funiculosum

Figure 3

Influence of fungal endophytes on (a) Relative water content (b) Melondialdehyde of rice under stress [S] and without stress [WS].

± indicates standard error of mean (n = 4); the dissimilar letters indicate significant difference at P < 0.05 by using Duncan's Multiple Range Test.
C: control (uninoculated plants); Cl: Ceriporia lacerate and En: Endomelanconiopsis endophytica, Pf: Penicillium funiculosum

**Figure 4**

Influence of fungal endophytes on Photosynthetic pigments (a) Chlorophyll a (b) Chlorophyll b (c) Total Chlorophyll (d) Carotenoids (e) Chlorophyll a/b ratio of rice under stress [S] and without stress [WS]. ± indicates standard error of mean (n = 4); the dissimilar letters indicate significant difference at P < 0.05 by using Duncan's Multiple Range Test

C: control (uninoculated plants); Cl: Ceriporia lacerate and En: Endomelanconiopsis endophytica, Pf: Penicillium funiculosum

**Figure 5**


**Supplementary Files**

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- 3.Tables.pdf
- 4.SM.pdf