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Fungal endophyte mediated salinity stress tolerance in rice (*Oryza sativa* L.)

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Abstract
The soil salinization has led to degradation of agricultural soil that exhibit negative impact on crop growth and production. The endophytes aid their host to survive in harsh environmental conditions by adapting to environment along with host plant. With this background the study was conducted to evaluate fungal endophytes isolated from the plants of North western Himalayan region to impart salinity stress tolerance in rice. Forty-eight fungal endophytes were screened for salt stress tolerance using different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 M) of NaCl. Among which four were found to be salt tolerant. They were identified as *Ulocladium* sp., *Fusarium avenaceum*, *Chaetomium* sp. and *F. tricinctum* by ITS region sequence homology. Under *in-vitro* condition the fungal endophytes inoculated rice seedlings recorded increased growth compared to uninoculated plants under salinity stress. The endophytes inoculated plants showed increased growth, yield and physiological traits compared to uninoculated plants under greenhouse conditions. Among the four endophytes evaluated, the *Fusarium avenaceum* showed superior in imparting salt stress besides improving the growth and yield of rice.

Keywords: North western Himalaya, fungal endophytes, salinity stress, rice, its region

Introduction
Salinity is one of the major abiotic stresses that poses great challenge in crop production all over the world. It results in significant reduction in plant growth and end up in crop failure through both osmotic stress and ionic toxicity (Wang *et al*., 2018) [1]. It is estimated that by 2050 around 50 per cent of all arable land (~1 billion ha) worldwide will be affected by salinity, which represents about 7 per cent of the earth's continental area. It has been estimated that worldwide 20 per cent of total cultivated and 33 per cent of irrigated agricultural land is affected by high salinity (Shrivastava and Kumar, 2015) [2]. Central Soil Salinity Research Institute (CSSRI) has estimated that about 1.7 mha land is affected by salinity.

Salinization of soil is contributed by excessive accumulation of prominent ions such as sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), sulphates (SO_4^{2-}) and chlorides (Cl^-) in soil. Soil is defined as being saline when the electrical conductivity (EC) of the saturation extract (ECe) in the root zone exceeds 4 dSm^{-1} at 25 ºC, exchangeable sodium percentage less than 15 per cent (w/v) and pH not exceeding 8.5, inhibiting plant growth and cellular functions. The most abundant ion in salt-affected soils is Na^+ and hence the exchange phase is dominated by Na^+ ions (Munns and Tester, 2008) [3].

Rice (*Oryza sativa* L.) is a major staple food crop in Asia. It is the most sensitive cereal to salinity stress followed by durum wheat (*Triticum turgidum* ssp. durum) and bread wheat (*Triticum aestivum*) (Munns and Tester, 2008) [3]. Rice is very sensitive to salinity at the early vegetative stage and later reproductive stages. (Ghosh *et al*., 2016) [4]. To make crop salinity tolerant by means of conventional breeding through wide hybridization is more labour intensive and time-consuming approach. In recent years, use of endophytes to impart salinity stress tolerance in crop plant is paving the way for resilient agriculture. It is well established fact that all plants are associated with microorganisms mainly bacteria and fungi that reside inside plant tissue and inducing stress tolerance. The fungal endophytes belong to class II provide an advantage of habitat specific stress tolerance to the host plants (Rodriguez *et al.*, 2008) [5].

The salinity stress tolerance is mainly based on the ability of the plant to keep the cytosolic Na^+ concentration as low as possible. The fungal endophytes reduce the Na^+ ion by reducing expression of antiporters thus reducing the cytoplasmic Na^+ ion concentration which enable plants to acquire more water (Sampangi-Ramaiah *et al*., 2020) [6]. The cytoplasmic Na^+ levels can also be kept low by exporting Na^+ into the vacuole (Molina-Montenegro *et al*., 2020) [18].

The Endophytes also produce antioxidant enzymes, to scavenge reactive oxygen species produced in response to salinity, and phytohormones, that promote plant growth (Manasa *et al*., 2020) [7]. This study aimed at examining the fungal endophytes isolated from the plants in cold desert of North western Himalayan region for their ability to impart salt tolerance in salt sensitive rice variety IR-64.

Material and Methods

The forty-eight fungal endophytes isolated from North Western Himalaya which were conserved in the School of Ecology and Conservation Laboratory, Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bengaluru were collected and rejuvenated by sub-culturing on Potato Dextrose Agar (PDA) were used for screening against salinity stress.

Screening of fungal endophytes for salt tolerance

The hyphal disc (0.9 mm diameter) of 5 days old fungal cultures was inoculated on potato dextrose agar (PDA) supplemented with different concentrations of sodium chloride (0.5, 1.0, 1.5, 2.0 and 2.5 M) and the control was maintained without sodium chloride. After 5 days of incubation at 30±2 °C, the radial growth of fungal mycelia was recorded by measuring its colony diameter (cm). The lethal NaCl concentration at which colony growth has reduced to 50 per cent over the respective control (LC_{50}) was calculated by probit analysis (Bekker *et al.*, 2006) [8]. The fungal endophytes having 50 per cent growth reduction at higher NaCl concentration and able to grow at 2.5 M NaCl were selected as salt tolerant fungal endophytes.

Standardization of salinity stress tolerance in rice

The uniform sized rice seeds (IR-64 salinity sensitive variety) were surface sterilized (Arnold *et al*., 2000) [9] and germinated. The salinity stress was imposed by soaking germination papers in different NaCl concentration solutions (25, 50, 75, 100, 125, 150, 175 and 200mM) for 30 minutes and then pre-germinated seeds were placed on the germination paper and incubated at 25 °C in growth chamber. Sterile water was used for control. Seedling length was recorded after 14 days and LC_{50} value for NaCl concentration was determined by the probit analysis (Bekker *et al.*, 2006) [8].

***In vitro* evaluation of selected fungal endophytes against salt stress in rice**

The mycelial suspension of five days old selected fungal isolates was prepared using sterile distilled water and gently scraping the mycelial mat grown on PDA with the help of paint brush (Dhingra and Sinclair, 1993) [10]. The inoculum load was adjusted to ~10^4 CFU/mL by diluting with sterile distilled water using micrometry. The pre-germinated rice seeds were incubated with fungal suspension for 3 h for effective colonization followed by washings with sterile distilled water to remove fungal hyphal bits on seed surface. The inoculated pre-germinated seeds were placed on set of germination papers soaked in 150 mM NaCl solution (LC_{50} value) for 30 min to induce salinity stress. Control was maintained using sterile distilled water. Shoot and root length of seedlings were recorded after 14 days (Sangamesh *et al*., 2018) [11].

Re-isolation and confirmation of inoculated fungal endophytes from the seedlings

The leaf, stem and root were cut into small bits and were surface sterilized (Arnold *et al*., 2000) [9]. The plant excises were placed on potato dextrose agar (PDA) medium and incubated at 30 °C for 3 days. The emerged fungal colony were sub-cultured and confirmed as same by comparing with the mother culture.

Identification of selected endophytes

The four fungal isolates were identified based on the morphological characters (colony, fruiting body and spore characters) followed by molecular approach using ITS sequence. The genomic DNA was extracted from the fungal mycelium using the cetyl trimethyl ammonium bromide (CTAB) method. The ITS region was amplified by using universal primers, ITS_1 and ITS_4 (White *et al*., 1990) [12]. The sequences obtained were searched for homology using NCBI BLAST program (http://blast.ncbi.nlm.nih.gov; default parameters). The identification of the fungal endophytes was done based on maximum score and query coverage in the BLAST results (Manasa *et al*., 2015) [13].

Evaluation of fungal endophytes on rice against salinity stress under greenhouse conditions

The selected fungal endophytes inoculated rice seeds were transferred to pro-trays containing autoclaved coir pith and allowed to grow for 15 days then transplanted in main pots. The soil physico-chemical properties like soil pH, electrical conductivity (dS/m), available K_2O (kg /ha) and exchangeable Na^+ (meq/L) were analysed using standard protocol prior to the experiment. The salts needed to impose salinity was calculated and the plants were subjected to salt stress of 4 dS/m during grand growth stage *i.e*., 24 days after transplanting following Karnal method (Tomar and Minhas, 2004) [14] for 20 days. Observations for growth, yield and physiological parameters were recorded at different intervals.

Statistical analysis

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

Results and Discussion

Screening of fungal endophytes for salinity stress tolerance

Out of forty-eight fungal endophytes screened for salinity tolerance under laboratory conditions, four isolates (P-82, P-39, P-31 and P-10) found to be salt tolerant. However, the isolate P-39 was found superior as it could grow at 2.5 M NaCl concentration (Fig 1). Therefore, these four fungi were selected for further evaluation and characterization. Shoaib *et al.* (2018) [15] suggests two primary mechanisms of fungal tolerance to high salt concentrations, first is osmotic effect and second is specific ion effects. Microorganisms adapt to salinity stress by accumulating inorganic (potassium cations) osmolytes and organic (proline and glycine betaine) which is high energy demanding process that can result in reduced mycelial growth. Therefore, the increase in fungal growth at low solute concentration might be due to selective accumulation of solute to counter the increase in osmotic pressure. While in increased concentration of solute with an increase in osmotic potential, which results in exosmosis and can affect fungal growth through plasmolysis, but these fungi can regulate the osmatic stress. Therefore, these four fungi were selected for further evaluation and characterization.

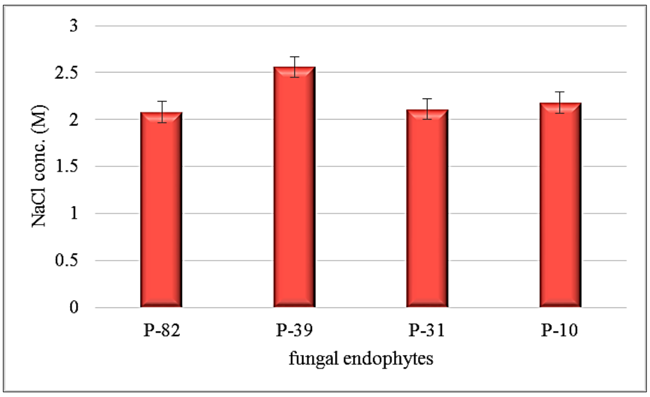


Fig 1: Colony growth of the salt tolerant fungal endophytes

Identification of salt tolerant fungal endophytes

Based on Internal transcribed Spacer (ITS) sequence homology the isolates were identified as P-82 (*Ulocladium* sp.), P-39 (*Fusarium avenaceum*), P-31 (*Chaetomium* sp.) and P-10 (*F. tricinctum*). Manasa *et al*. (2015) [13] identified the fungal OTUs by using ITS of the genomic DNA because the ITS region has the highest probability of successful identification for the broadest range of fungi with the most clearly defined gap between inter and intra specific variation in similar study.

Standardization of salinity stress tolerance in rice

The rice seedling length recorded 33.04 cm in control and 10.96 cm at 200 mM NaCl concentration. LC_{50} value was found 150mM. The reduced seedling length may be attributed to high salt concentration that increases osmotic potential, that in turn lowers water potential which must have reduced the ability of the plant to absorb water and nutrients that lead to disruption of the cell growth and development resulting in stunted growth (Munns and Tester, 2008) [3]. Similar results were recorded by Manasa *et al*. (2020) [7] in rice.

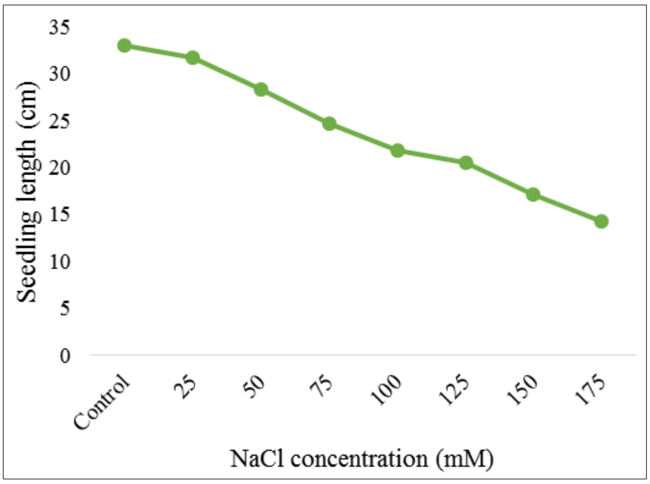


Fig 2: Effect of different concentrations of NaCl on rice seedlings

In-vitro evaluation of salt stress tolerant endophytic fungal isolates to impart salt stress tolerance to rice

Under salinity stress (Fig 3b) rice seedlings inoculated with salt tolerant fungal endophyte *Fusarium avenaceum* recorded significantly higher shoot length (12.50 cm) and root length (15.65 cm) that significantly differ from un-inoculated seedlings (7.52 cm 14.46 cm). The enhanced plant growth of inoculated plants may be attributed to the maintaining a low $Na^{+}:K^{+}$ ratio, upregulation of host stress responsive gene and by synthesis of host stress responsive proteins and hormones (Manasa *et al*., 2020) [7]. Since the endophytes are isolated from saline habitat their effect was pronounce in treatment under salinity stress rather among the treatments without salinity stress. Similar results were observed by Sampangi-Ramaiah *et al*., (2020) [6], Salt-tolerant endophyte (*Fusarium* sp.) isolated from salt-adapted Pokkali rice enhanced the growth of IR-64 rice variety under salt stress.

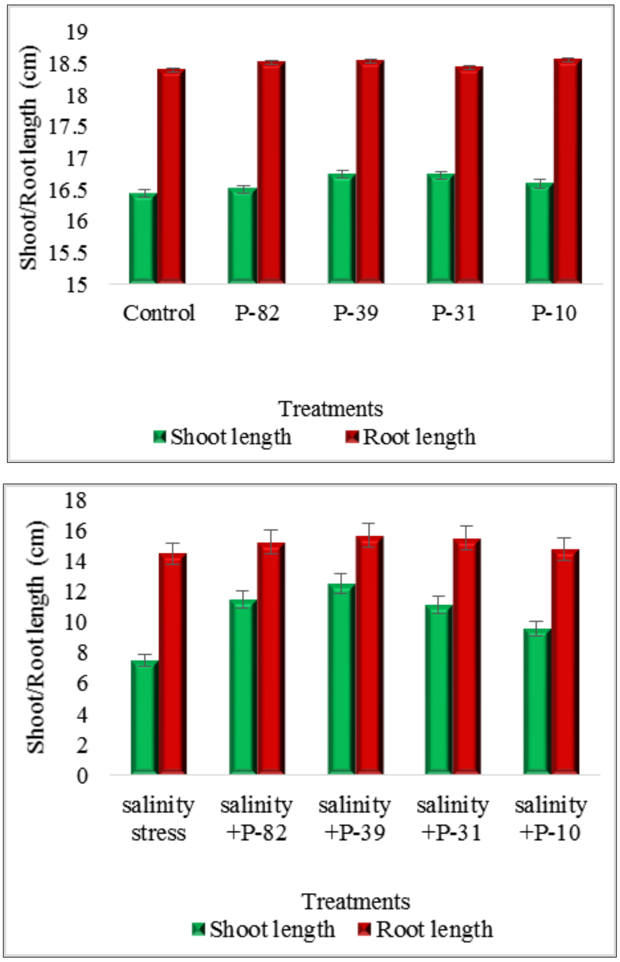
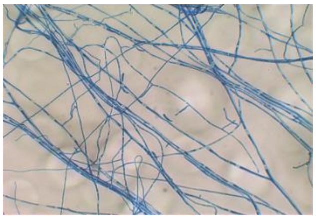
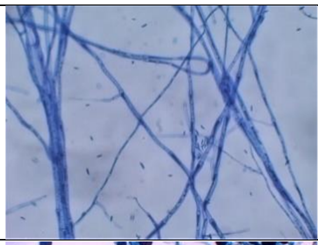
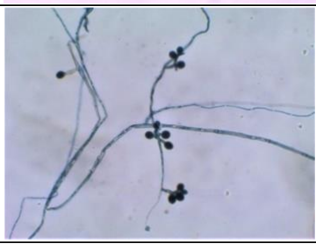


Fig 3: Effect of inoculation of fungal endophytes on seedling growth of Rice without (a) and with salinity stress (b)

Re-isolation of inoculated fungal endophytes from the seedlings

The inoculated fungi were reisolated along with endogenous endophytes from root stem and leaf. Their morphology was compared with morphology of mother culture of inoculated fungi (Fig 4) revealing that the fungi has colonized the seedling without disturbing the native endophytes. In similar studies (Sampangi-Ramaiah *et al*., 2020; Manasa *et al*, 2020) [6, 7], reisolated the inoculated the fungal endophytes from inoculated seedlings for the confirmation of the colonization.

P-39			Macro and micro conidia
P-10			Micro conidia
P-82			Septated spores
P-31			Lemon shaped spores

Effect of inoculation of fungal endophytes on growth of rice under salt stress

The fungal endophyte *Fusarium avenaceum* inoculation significantly increased the plant height, number of leaves and number of tillers compared to un-inoculated plants under salt stress (Table 1). Increased plant growth might be attributed to production of growth hormones, ion homeostasis and antioxidants production by the fungal endophytes under salinity stress which scavenges the reactive oxygen species (ROS) produced due to salinity and enhance the plant growth (Manasa *et al.*, 2020) [7]. Sampangi-Ramaiah *et al*. (2020) [6] reported that salt-tolerant endophyte isolated from salt-adapted Pokkali rice, a *Fusarium* sp., colonized the salt-sensitive rice variety IR-64, enhanced its growth under salt stress and conferred salinity stress tolerance with and lower Na^+/K^+ content in plant tissues.

Table 1: Effect of fungal endophytes on growth parameters of rice under salinity stress

Treatments	Plant height (cm)				Number of Tillers				No. of leaves			
	30 DAT	60 DAT	90 DAT	120 DAT	30 DAT	60 DAT	90 DAT	120 DAT	30 DAT	60 DAT	90 DAT	120 DAT
Control	33.20^{b}	62.20^{c}	84.80^{c}	85.40^{c}	1.40	20.00^{b}	28.40^{d}	24.60^{c}	8.00^{c}	82.80^{c}	113.40^{d}	98.40^{c}
Salt stress	23.20^{d}	48.40^{g}	58.20^{f}	66.20^{f}	1.80	12.60^{d}	15.20^{g}	24.60^{f}	8.80^{bc}	50.40^{f}	60.80^{g}	46.40^{f}
Ulocladium sp.	34.80^{a}	71.20^{a}	90.20^{a}	91.80^{ab}	2.20	23.60^{a}	33.20^{a}	27.60^{a}	11.00^{a}	96.40^{a}	132.8^{a}	110.4^{a}
F. avenaceum	34.60^{a}	70.20^{a}	89.40^{a}	92.80^{a}	2.20	22.60^{a}	31.60^{b}	26.00^{bc}	10.60^{a}	91.20^{b}	126.4^{b}	104.0^{b}
Chaetomium sp.	33.60^{ab}	68.20^{b}	88.00^{b}	90.80^{b}	2.40	22.00^{a}	29.80^{c}	25.60^{bc}	10.40^{a}	84.80^{c}	119.2^{c}	102.4^{bc}
Salt stress+*Ulocladium* sp.	26.20^{c}	56.80^{e}	64.80^{e}	70.00^{e}	2.20	17.20^{c}	17.60^{ef}	25.60^{de}	10.60^{a}	68.80^{de}	70.40^{f}	60.80^{de}
Salt stress +*F. avenaceum*	26.80^{c}	60.20^{d}	66.80^{d}	71.60^{d}	2.40	17.60^{c}	18.80^{e}	25.68^{d}	10.60^{a}	70.40^{d}	75.20^{e}	64.80^{d}
Salt stress+*Chaetomium* sp.	25.60^{c}	55.00^{f}	64.20^{e}	69.60^{e}	2.20	16.40^{c}	16.80^{f}	26.10^{e}	9.60^{ab}	65.60^{e}	67.20^{f}	57.60^{e}
CD@5%	1.20	1.33	1.25	1.22	NS	1.66	1.24	1.24	1.49	3.70	3.55	4.96

Note: Mean values followed by same super script in each column do not differ significantly at p=0.05 level by Duncan Multiple Range Test (DMRT)

Table 2: Effect of fungal endophytes on yield parameters of rice under salinity stress

Treatments	No of panicles	No. of seeds /plant	Seed yield(g) /plant
Control	9.20^{b}	564.53^{c}	12.40^{c}
Salt stress	6.20^{c}	262.17^{f}	5.24^{f}
Ulocladium sp.	12.00^{a}	790.91^{a}	17.30^{a}
F. avenaceum	12.00^{a}	796.56^{a}	17.40^{a}
Chaetomium sp.	11.40^{a}	722.13^{b}	15.90^{b}
Salt stress+*Ulocladium* sp.	8.60^{b}	384.56^{e}	7.69^{e}

Salt stress +*F. avenaceum*	8.80b	410.83d	8.22d
Salt stress+*Chaetomium* sp.	8.40b	382.85e	7.66e
CD@5%	1.20	19.75	0.35

Note: Mean values followed by same super script in each column do not differ significantly at *p*=0.05 level by Duncan Multiple Range Test (DMRT)

The *Fusarium avenaceum* treated plants showed significant increase in yield parameters (Table 2), the enhanced yield parameter may be attributed to the sustained photosynthetic process in inoculated plants. Inoculation of fungal endophytes reduce uptake of Na^+ ions due to upregulation of plant response genes, increased phytohormone production, enhanced antioxidant enzyme activity might have resulted in reduced ROS production which would oxidize the photosynthetic pigment otherwise (Sampangi-Ramaiah *et al*., 2020) [6]. These combinedly resulted in higher photosynthetic efficiency leading to higher yield parameters than uninoculated plants under salinity stress where the ROS produced in response to salinity would oxidise photosystem and reduce photosynthetic efficiency (Munns and Tester, 2008) [3]. Jogawat *et al*. (2013) [16] reported that the *Piriformospora indica* inoculated rice plants resulted in higher plant growth, biomass and yield attributes.

The Chl a, Chl b, total Chl and carotenoid contents (Table 3) reduced in salinity stress condition, may be due to the destruction of pigment by reactive oxygen species (ROS) produced as result of increased salinity (Jogawat *et al*., 2013) [16]. The *Fusarium avenaceum* inoculated plant recorded significantly higher Chl a, Chl b, total Chl and carotenoid content may be due to antioxidants which neutralizes the ROS thus protecting the photosystems (Sampangi-Ramaiah *et al*., 2020) [6].

Table 3: Effect of fungal endophytes on physiological parameters of rice under salinity stress

Treatments	Chl a (mg/g FW)	Chl b (mg/g FW)	Total chl (mg/g FW)	Carotenoid content (mg/g FW)	RWC (%)	Proline content (µmol/g FW)
Control	0.75c	0.30f	0.90d	0.31b	85.62b	5.32e
Salt stress	0.58g	0.30f	0.73h	0.25c	83.84d	10.22d
Ulocladium sp.	0.78a	0.35a	0.92a	0.34a	85.75a	5.32e
F. avenaceum	0.78a	0.35a	0.93a	0.35a	85.76a	5.33e
Chaetomium sp.	0.77b	0.32d	0.91c	0.31b	85.72a	5.32e
Salt stress+*Ulocladium* sp.	0.60e	0.33c	0.77f	0.27c	84.25c	11.85b
Salt stress +*F. avenaceum*	0.62d	0.34b	0.81e	0.30b	84.76b	11.94a
Salt stress+*Chaetomium* sp.	0.59f	0.31e	0.75g	0.26c	84.00c	11.33c
CD@1%	0.007	0.009	0.008	0.024	0.24	0.021

Note: Mean values followed by same super script in each column do not differ significantly at *p*=0.05 level by Duncan Multiple Range Test (DMRT)

FW: Fresh weight, RWC-Relative water content

The *Fusarium avenaceum* inoculated plants recorded significantly higher relative water content while under salinity without endophytes inoculated plants had lower RWC. The reduced RWC in uninoculated plants may be due to reduced ability of plant to uptake water by the roots attributing to higher osmotic pressure by enhanced salinity at root surface. (Jogawat *et al*., 2013) [16]. While ion homeostasis and accumulation of organic solutes, soluble sugars, proteins by fungal endophytes might enhance the water uptake and lead to higher RWC (Bagheri *et al*., 2013) [17].

The *Fusarium avenaceum* inoculated plants recorded significantly higher proline content (Table 3) under salt stress. Proline helps in protecting against ROS produced under salinity stress. Bagheri *et al*. (2013) [17] reported higher proline, total proteins and sugars in *Piriformospora indica* colonized rice plants under salt stress. Similar results were observed by Manasa *et al*. (2020) [7] wherein *Fusarium oxysporum* a fungal endophyte from habitat adapted plant confers habitat-specific salinity stress tolerance in saline sensitive paddy variety IR-64 by proline accumulation.

Conclusion
Some of the fungal endophytes isolated from plants of north western himalayan region have salinity stress tolerance. The *Fusarium avenaceum* was found superior to other fungal endophytes in inducing salinity stress tolerance to salt sensitive rice under *in-vitro* and pot experiment. Thus, endophytes can play a crucial role in salinity stress tolerance in crops.

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