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Fungal Endophyte Mediated Salinity Stress Tolerance in Mung Bean (*Vigna radiata* L. Wilczek)

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Arunkumar G. Pandit : Designing of experiment & manuscript prepration;
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Abstract

The soil salinization has led to degradation of agricultural soils that negatively impact crop growth and production. Crops which are sensitive to salinity gets affected severely by soil salinity. The endophyte 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 the fungal endophytes isolated from the plants of North western Himalayan region to impart salinity stress tolerance in mung bean. Out of forty-eight fungal endophytes screened four were salt tolerant and they were identified as *Ulocladium* sp., *Fusarium avenaceum*, *Chaetomium* sp. and *F. tricinctum*. The fungal endophytes *Fusarium avenaceum* found superior over other endophytes in imparting salinity stress tolerance in Mung bean. The effect of fungal endophytes on seedling growth was more pronounced under salinity stress condition than in non-stress conditions. Under pot experiment the plants inoculated with *Fusarium avenaceum* recorded higher plant growth, yield and physiological parameters. Thus, the fungus was found as potential endophyte for imparting salinity stress tolerance in mung bean.

Keywords : North western Himalaya, Fungal endophytes, Salinity stress, Mung bean, ITS region.

Abiotic and biotic stresses substantially reduce the plant growth, vigour, yield and nutritional status. Salt stress is one of the important and widespread abiotic stress, that have visible effects related to various growth parameters of the plants (Gull and Kausar, 2019). It is estimated that by 2050 around 50 per cent of all arable land (~1 billion ha) world wide will be impacted 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 2014). Salinity affects plants at two levels, initial osmotic effect; increased concentration of salts at soil-root interface enhances osmotic potential that lowers water potential which reduces the ability of the plant to absorb water and nutrients from the soil, followed by ionic stress when salt accumulation reaches its toxic level (Munns and Tester, 2008).

Endophytes are the organisms, often bacteria or fungi, that invade and thrive in plant tissues without causing any apparent disease symptoms. The shorter generation period of endophytes makes them forerunner in acquiring adaptation to stress under severe selection pressure than their host which are having a lengthy generation period. They also help their host to survive under stress conditions. This is referred to as habitat adapted symbiosis (Rodriguez *et al*., 2008). Though many efforts are being made to induce plant tolerance to abiotic stress through conventional and molecular approaches, the unconventional approach that is using plant microbiome is recently gaining attention in research (Ravikanth *et al*., 2017).

The endophytes have ability to reduce deleterious effect of salt stress on host plant by scavenging reactive oxygen species (ROS) through antioxidant enzymes, ion homeostasis, salt compartmentalisation, accumulation of organic solutes, soluble sugars, proteins, lipids *etc* (Bagheri *et al.,* 2013). Recent studies suggested that, the use of endophytic fungi play a crucial role in plant growth promotion resulting in higher yield and increased resistance to abiotic stresses (Ikram *et al*, 2019; Manasa *et al*., 2020; Sampangi-Ramaiah *et al*., 2020).

Mung bean (*Vigna radiata* L. Wilczek) has been grown during kharif season. Despite the high prices of pulses, large gap between potential and actual yield of pulse crops are lower than the major competing crops. As a result, pulses are mainly grown on marginal land and remote farm areas which are subjected to vagaries of the climate and soils. Growing pulses in such conditions results in large fluctuations in production (Mehandi *et al*., 2019). Therefore, it necessitates improvement in abiotic stress tolerance in mung bean. In this study, a set of fungal endophytes were examined for their ability to impart salinity stress tolerance in mung bean variety KKM-3 which is sensitive to salt stress.

MATERIAL AND METHODS

Collection of Fungal Endophytes

Forty-eight fungal endophytes isolated from North Western Himalaya and 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). These fungi were used for screening against salinity stress.

Screening of Fungal Endophytes for Salinity Stress Tolerance

The hyphal disc (0.9 mm diameter) of 5 days old fungal cultures was inoculated on 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 addition of 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 was reduced to 50 per cent over the respective control (LC50) was calculated by probit analysis (Bekker *et al.*, 2006). The fungal endophytes having 50 per cent growth reduction at higher NaCl concentration and capable to grow at 2.5 M NaCl were selected as salt tolerant.

Standardization of Salinity Stress Tolerance in Mung Bean

The uniform sized mung bean seeds (KKM-3) were surface sterilized (Arnold *et al.,* 2000) and germinated. The salinity stress was imposed by soaking in the germination papers at different concentration of NaCl solutions (25, 50, 75, 100, 125, 150, 175 and 200 mM) 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 6 days and LC50 value for NaCl concentration was determined by probit analysis (Bekker *et al*., 2006).

In-vitro Evaluation of Selected Fungal Endophytes Against Salt Stress in Mung Bean

The mycelial suspension of 5 days old selected fungal isolates was prepared in sterile distilled water (Dhingra and Sinclair, 1995). The inoculum load (propagules) was adjusted to ~10^4 CFU/ml using micrometry by diluting with sterile distilled water. The pre-germinated mung bean seeds were incubated with fungal suspension for 3h for effective colonization followed by washing with sterile water to remove fungal hyphal bits on seed surface. The inoculated pre-germinated seeds were placed on set of germination papers soaked in 112 mM NaCl solution (LC50 value) for 30 min to induce salinity stress. Control was maintained using sterile distilled water. Shoot and root length of seedlings were recorded after 6 days (Sangamesh *et al*., 2018).

Confirmation of Fungal Endophytes in Inoculated Seedlings

The leaf, stem and root were cut into small bits and surface sterilized (Arnold *et al*., 2000). These plant excises were placed on PDA medium and incubated at 30 °C for 3 days. The emerged fungal colonies were sub-cultured and confirmed whether or not they are same by comparing with respective 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 region 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). 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).

Evaluation of Fungal Endophytes on Mung Bean Against Salinity Stress Under Greenhouse Conditions

The selected fungal endophytes inoculated mung bean seeds were transferred to 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 by 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 sowing) by following Karnal method (Tomar and Minhas, 2004) for 20 days. The Complete Randomized Design (CRD) was used for the pot experiment with five replication and two plants in each replication. Observations for growth, yield and physiological parameters were recorded at different intervals.

Statistical Analysis

The experiment was conducted using completely 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 Isolates to Salinity Stress Tolerance

Among the 48 fungal endophytes screened for salinity stress tolerance, four isolates (P-82, P-39, P-31 and P-10) were found saline tolerant (Fig 1). However, *Fusarium avenaceum* found superior over other endophytes. The colony growth of fungi reduced as the salt concentration increased. This indicated that the limit of salt tolerance of the different isolates. Shoaib *et al.* (2018) reported that the two primary mechanisms of fungal tolerance to high salt concentrations; first is osmotic effect involves accumulation inorganic (potassium cations) osmolytes and organic (proline and glycine betaine) osmolytes which is high energy demanding process that can result in reduced mycelial growth. There fore, the increase in fungal growth at low solute concentration might be due to selective accumulation of solute to counter the increase in osmotic pressure. Second one is specific ion effect, with increased

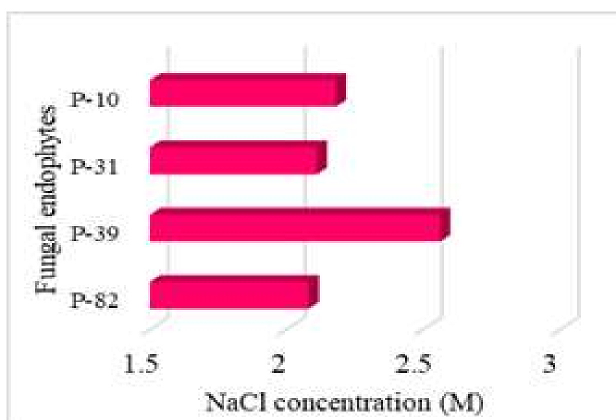

Fig. 1 : LC50 value and radial growth of the salt tolerant fungal isolates

concentration of solute with an increase in osmotic potential, results in exosmosis and can affect fungal growth through plasmolysis, but these salt tolerant fungi can regulate the osmatic stress. Therefore, such four fungi were selected for further evaluation and characterization.

Identification of the Four-Salt Tolerant Fungal Isolates

Internal transcribed spacer (ITS) region of 18S ribosomal gene has highest probability of successful identification for the broadest range of fungi with the most clearly defined gap between inter and intra specific variations (Manasa *et al*., 2015) and therefore, in the present study the salt tolerant fungal endophytes were identified using ITS region sequence homology. Based on ITS sequence homology the four isolates were identified as *Ulocladium* sp. (P-82), *Fusarium avenaceum* (P-39), *Chaetomium* sp. (P-31) and *F. tricinctum* (P-10).

Evaluation of Salinity Stress Tolerance in Mung Bean

The seedling length of mung bean decreased with increased concentration of NaCl (Fig.2). The lethal concentration of NaCl for 50 per cent growth reduction (LC_{50}) was found to be 112 mM. The reduced seedling length may be attributed to high salt concentration which increases osmotic potential. In turn lower water potential reduces the ability of plant to absorb water and nutrients that lead to disruption of the cell growth

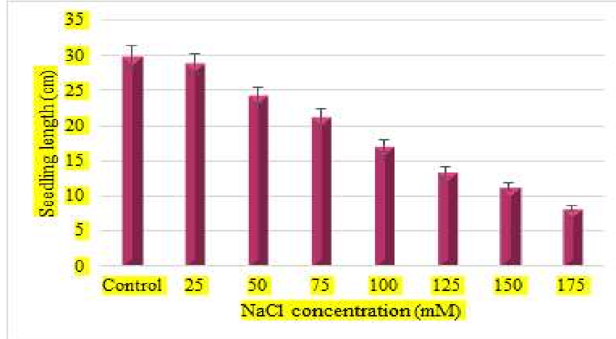

Fig. 2 : Effect of different concentrations of NaCl on seedling growth of mung bean

and development (Munns and Tester, 2008). Similar results were also recorded by Ali *et al*. (2021) in mung bean.

***In-vitro* Evaluation of Salt Stress Tolerant Endophytic Fungal Isolates to Impart Salt Stress Tolerance to Mung Bean.**

Under salinity stress, the salt tolerant fungal endophyte *Fusarium avenaceum* inoculated plants recorded higher shoot and root length that significantly differ from un-inoculated seedlings (Fig 3). Increased plant growth of endophyte inoculated plants may be attributed to maintaining low Na^{+} : K^{+} ratio, upregulation of host stress responsive gene and synthesis of host stress responsive proteins and hormones (Manasa *et al*., 2020). Since the endophytes are isolated from harsh habitat and their effect was pronounced under salinity stress compared to non-stress plants. Ali *et al*. (2021) reported that the

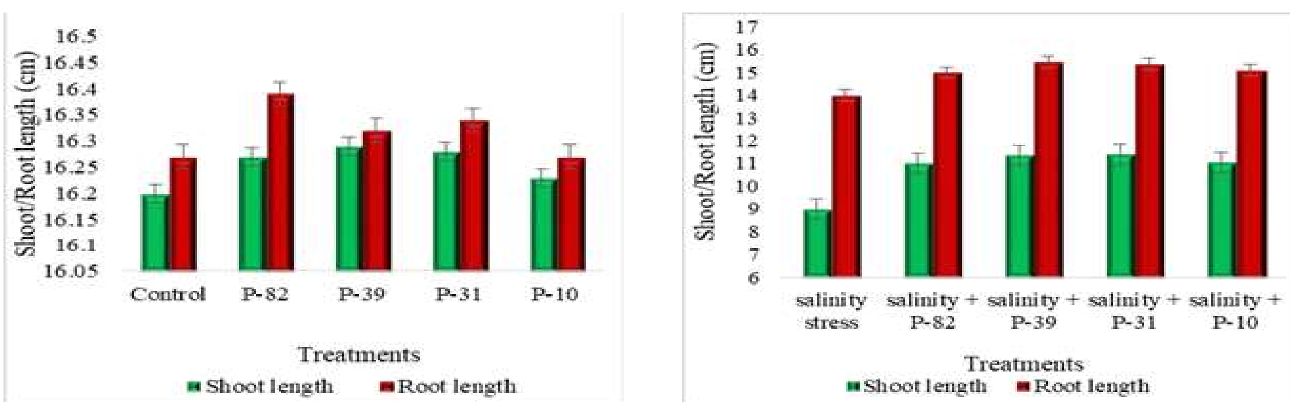

Fig. 3 : Effect of inoculation of endophytic fungal isolates on seedling growth of mung bean without salinity stress (a) and with salinity stress (b)

fungal endophyte (*Aspergillus awamori*) associated with Mung bean seedlings exhibited enhanced seedling growth due to ion homeostasis and antioxidant enzyme activity which resulted in low accumulation of stress markers. Higher concentration of Ca, Mg, K, N, and P also mitigate the salt stress.

Confirmation of Fungal Endophytes in Inoculated Mung Bean Seedlings

The inoculated fungi were recovered from root, stem and leaf bits of mung bean seedlings and their morphology were compared with their mother culture. These fungi revealed morphologically similar characters of the mother culture (Fig. 4) and therefore they were confirmed as same isolates (Manasa *et al.*, 2020 and Sampangi-Ramaiah *et al.*, 2020).

Effect of Inoculation of Fungal Endophytes on Growth of Mung Bean Under Greenhouse Conditions

Increased plant height was recorded in endophyte inoculated plants (Table 1). The enhanced plant height in inoculated plants under salinity stress may be due to regulation of ion homeostasis, accumulation of compatible solutes and phytohormone production by endophytes (Ali *et al.*, 2021) this upheld the plant

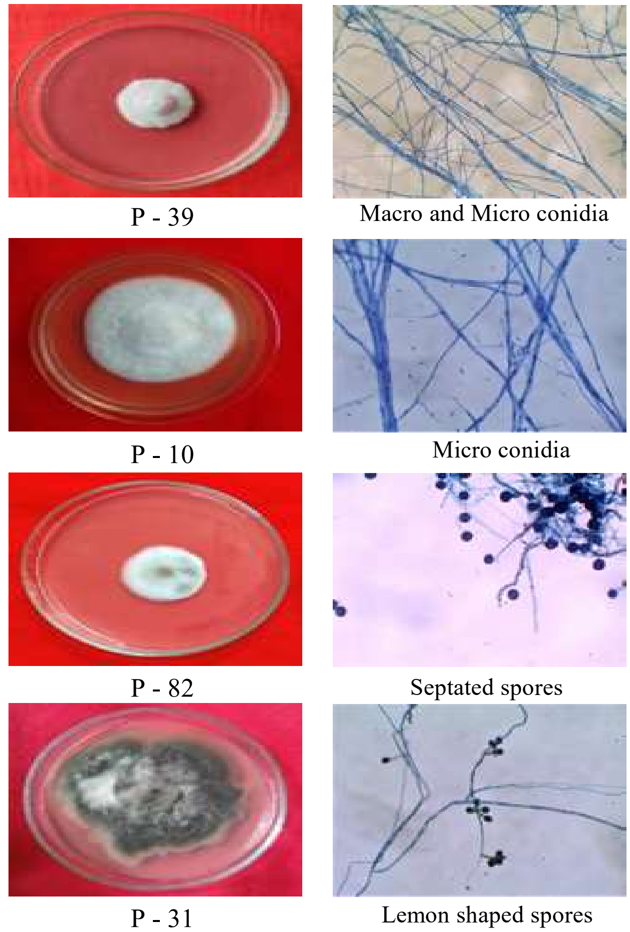

Fig. 4. Colony and microscopic view (40X) of spores of salt stress tolerant endophytic fungal isolates

TABLE 1

Effect of fungal endophytes on growth parameters of mung bean under salinity stress

Treatments	30 DAS			60 DAS		
	Plant height (cm)	No. of branches	No. of leaves	Plant height (cm)	No. of branches	No. of leaves
Control	22.20 ^{cd}	2.00	6.00	28.20 ^{cd}	4.40 ^{d}	13.20 ^{d}
Salt stress (4 dS/m)	18.40 ^{ef}	2.00	6.00	23.20 ^{g}	3.58 ^{g}	10.74 ^{g}
Ulocladium sp.	23.00 ^{bc}	2.00	6.00	30.70 ^{b}	4.80 ^{b}	14.40 ^{b}
F. avenaceum	24.40 ^{a}	2.00	6.00	32.30 ^{a}	5.00 ^{a}	15.00 ^{a}
Chaetomium sp.	23.60 ^{ab}	2.00	6.00	30.40 ^{b}	5.00 ^{a}	15.00 ^{a}
F. tricinctum	23.20 ^{abc}	2.00	6.00	28.40 ^{cd}	4.60 ^{c}	13.80 ^{c}
Salt stress+ *Ulocladium* sp.	20.40 ^{e}	2.20	6.00	27.60 ^{de}	4.00 ^{f}	12.00 ^{f}
Salt stress + *F. avenaceum*	21.20 ^{de}	2.00	6.00	29.00 ^{c}	4.40 ^{d}	13.20 ^{d}
Salt stress + *Chaetomium* sp.	20.60 ^{e}	2.00	6.00	25.20 ^{f}	4.40 ^{d}	13.20 ^{d}
Salt stress+ *F. tricinctum*	20.40 ^{e}	2.00	6.00	26.70 ^{e}	4.20 ^{e}	12.60 ^{e}
CD@5%	1.30	NS	NS	0.91	0.17	0.52

Note : Mean values followed by same super script in each column do not differ significantly at p=0.05 level by DMRT fitness under salinity stress. Number of branches and leaves did not differ significantly at 30 days of growth but differences were observed in 60 days old plants indicating that the endophytes can influence the growth against salinity stress. However, the *Fusarium avenaceum* was found superior in enhancing the growth at salt stress compared to other three endophytes.

Further, the *Fusarium avenaceum* treated plants showed significant increase in yield parameters (Table 2). The enhanced yield parameter in endophyte inoculated plants may be attributed to sustained photosynthetic process. Inoculation of fungal endophytes reduces the uptake of Na^+ ions due to upregulation of plant response genes, increased phytohormone production, enhanced antioxidant enzymatic activity (Sampangi-Ramaiah *et al*., 2020). This indicated that the endophyte inoculation resulted in higher photosynthetic efficiency leading to higher yield parameters than uninoculated plants under salinity stress (Munns and Tester, 2008). Ikram *et al*. (2019) reported increased biomass and chlorophyll content in endophyte treated plants.

TABLE 2

Effect of fungal endophytes on yield parameters of mung bean under salinity stress

Treatments	No. of pods /plant	Weight of pods (g)/ plant	Seed yield (g) /plant
Control	21.60 b	15.94 c	12.79 c
Salt stress (4 dS/m)	20.20 c	6.86 h	5.14 g
Ulocladium sp.	22.40 a	16.39 ab	14.50 b
F. avenaceum	22.20 a	16.48 a	15.31 a
Chaetomium sp.	22.00 a	16.40 ab	14.07 b
F. tricinctum	22.00 a	16.27 b	13.91 b
Salt stress + *Ulocladium* sp.	22.60 a	9.44 e	8.90 de
Salt stress + *F. avenaceum*	22.80 a	9.73 d	9.55 d
Salt stress + *Chaetomium* sp.	22.20 a	9.07 f	8.65 e
Salt stress + *F. tricinctum*	22.20 a	8.88 g	7.89 f
CD @ 5 %	1.09	0.16	0.72

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 3

Effect of fungal endophytes on physiological parameters of Mung bean 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	0.92 b	0.26 d	2.11 d	0.29 b	89.47 c	7.03 g
Salt stress (4 dS/m)	0.81 f	0.21 f	1.83 i	0.24 d	85.90 f	12.35 e
Ulocladium sp.	0.92 b	0.29 b	2.14 b	0.35 a	91.16 b	7.05 f
F. avenaceum	0.93 a	0.30 a	2.16 a	0.37 a	91.84 a	7.05 f
Chaetomium sp.	0.92 b	0.28 c	2.13 c	0.30 b	89.82 c	7.04 fg
F. tricinctum	0.92 b	0.29 b	2.14 b	0.33 b	91.10 b	7.04 fg
Salt stress+ *Ulocladium* sp.	0.87 c	0.26 d	2.00 f	0.28 c	87.27 d	13.35 b
Salt stress + *F. avenaceum*	0.87 c	0.28 c	2.02 e	0.30 b	87.62 d	13.83 a
Salt stress + *Chaetomium* sp.	0.83 e	0.22 e	1.89 h	0.25 c	86.14 e	12.75 d
Salt stress+ *F. tricinctum*	0.85 d	0.26 d	1.96 g	0.26 c	86.45 e	13.14 c
CD@1%	0.007	0.008	0.009	0.034	0.60	0.027

Note : Mean values followed by same super script in each column do not differ significantly at p = 0.01 level by DMRT

FW-Fresh weight, RWC- Relative water content

The physiological parameters such as chl a, chl b, total chl and carotenoid content was significantly higher in *F. avenaceum* inoculated plants (Table 3). This further indicated the superiority of the fungus. Enhanced photosynthetic pigments in endophyte inoculated plants may be due to production of antioxidants by endophytes which could neutralize the ROS and thus protecting photosynthetic pigments. The chl a, chl b, total chl and carotenoid content was reduced in uninoculated plants under salinity stress. This is due to production of reactive oxygen species which oxidizes the photosynthetic pigments (Munns and Tester 2008). Ali *et al*., (2021) reported the protection of photosynthetic pigments by anti-oxidants and phytohormones produced by fungal endophytes.

Similarly, the plants inoculated with *Fusarium avenaceum* significantly increased the relative water content (RWC) compared to un-inoculated plants under salinity stress (Table 3). This may be due to ion homeostasis and accumulation of organic solutes, soluble sugars, proteins by fungal endophytes (Bagheri *et al*., 2013). While, the reduced RWC in uninoculated plants may be due to reduced water uptake by the roots attributed to higher salinity at root surface resulting in higher osmotic potential which leads to the reduced ability of plant to absorb water (Munns and Tester, 2008). Ali *et al*. (2021) reported that the plants under salinity stress without endophytes inoculation lowered RWC compared to inoculated plants. The *Fusarium avenaceum* inoculated plants recorded significantly higher proline content (Table 3) under salt stress. The proline provide protection against ROS produced under salinity stress. Bagheri *et al*. (2013) reported higher proline, proteins and sugars in *P. indica* colonized rice plants under salt stress.

The fungal endophytes isolated from plants of North Western Himalayan region showed salinity stress tolerance. Among them *Fusarium avenaceum* found superior in inducing salinity stress tolerance in mung bean under *in-vitro* as well as in pot experiment. The study revealed that the endophytes can impart salinity stress tolerance in Mung bean.

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