Transgenerational Persistence of Endophyte *Fusarium incarnatum* Induced Salt Stress Tolerance in Salt Sensitive Rice

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Abstract

Several studies have demonstrated the role of fungal endophytes in imparting tolerance to its host plants in alleviating abiotic stresses such as drought, high temperature and salinity. For such effects to be translated into viable crop improvement, it is to be proved that endophytes are not only vertically transmitted but also confer stress tolerance stably in subsequent sporophytic generation. In this study, we demonstrated this using a saline tolerant endophyte, *Fusarium incarnatum* in a salt sensitive rice variety, IR 64. In the first sporophytic generation, plants colonized by the fungi were significantly tolerant to salinity stress compared to those not colonized by the fungi. Seeds and seedlings obtained from the first sporophytic generation were found to be colonized with the fungi indicating that the fungus had been vertically transmitted through the seeds. Further more, the successive generation plants were also found to possess significant tolerance to salinity stress compared to those derived from control (un-inoculated) plants. This result is among a very few to demonstrate the persistence of an endophyte-induced trait in subsequent sporophytic generation in crop plants.

Keywords : Endophyte, *Fusarium incarnatum,* Vertical transmission, Salinity stress

Endophytes, both fungi and bacteria, are present ubiquitously in all plants and colonize different parts of host (Philipson and Christey, 1986). Localized to the intercellular spaces of plant cells, they maintain a symbiotic relationship with their host plants (Arnold and Lutzoni, 2007). Endophytes do not cause any disease symptoms in their host plants and are reported to play an important role in plant growth and development, help in nutrient acquisition and confer tolerance to abiotic and biotic stresses (Santoyo *et al.,* 2016; Chhabra & Dowling, 2017; Lata *et al.,* 2018 and Tasmiya & Earanna, 2021). Introduction of endophytes from stress adapted plants such as xerophytes and halophytes to non-native plants have often been shown to improve their fitness under stress, compared to plants not colonized by the endophytes (Rodriguez *et al.,* 2008). For example, endophytes impart tolerance to their host plants against herbivory, heat, salt, disease and drought stress (Redman *et al.,* 2011; Hamilton & Bauerle, 2012; Azad & Kaminskyj, 2016; Cosme *et al.,* 2016 and Sangamesh *et al.,* 2018).

The effect of endophytes on plant fitness is more pronounced under stressful environments compared to non-stressed conditions (Rho *et al.,* 2018). These studies have raised the possibility of using endophytes to alleviate and or mitigate crop plants from abiotic and biotic stresses.

The mechanism through which such endophyte induced stress tolerance is imparted is however little understood. One of the possibilities is the ability of endophyte to modulate host plant gene expression, especially those that are relevant in combating the given stress. Sampangi-Ramaiah *et al.* (2020) showed that rice plants colonized by a salt adapted *F. incarnatum* led to the upregulation of genes involved in abiotic and biotic stress tolerance, when subjected to salinity stress. Bajaj *et al.* (2018) showed that colonization of soybean plants by *Piriformospora indica* resulted in an upregulation of genes associated with phenyl-propenoid pathway and lignin pathway, both of which are known to play an important role in oxidative stress tolerance. In rice, *P. indica* confers drought tolerance by up-regulating miR159/miR396 that target MYB and GRF transcription factors, involved in regulation of growth and hyposensitivity response (Mohsenifard *et al.,* 2017). Yet another study in rice, showed that *P. indica* colonization led to the differential miRNA expression that targeted transcription factors involved in nutrient uptake, Na^+ transporters and growth regulators including auxin responsive proteins (Kord *et al.,* 2019).

While the prospects of using endophytes to mitigate crop abiotic stresses looks promising, there are only a very few applications in commercial agriculture. Among the various constraints mentioned by Chitnis *et al.* (2020), it is still unclear to what extent such endophyte mediated modulations are maintained in successive sporophytic generations. Any effort that demonstrates a beneficial impact of endophytes that is stably inherited to successive generations would provide a strong framework to look at endophytes as an alternative method towards development of crop resistance to abiotic and biotic stresses. Wheat florets, for instance, when sprayed with *Paraburkholderia phytofirmans* endophyte were shown to be localized in seed embryos and improve growth traits in progeny generations (Mitter *et al.,* 2017). In yet another study in wheat, seeds coated with *Penicillium* was shown to enhance plant biomass for three-generations compared to untreated seeds, when subjected to salinity stress (Vujanovic *et al.,* 2019). The endophyte, *Epichloe* induced proline accumulation in maternal and seedling generations of *Lolium multiflorum* plants when exposed to ozone stress (Ueno *et al.,* 2020). *Sphingomonas melonis* was able to transfer across generations and conferred disease tolerance in rice by producing anthranilic acid which interferes with sigma factor RpoS of the seed-borne pathogen *Burkholderia plantarii*, probably leading to impairment of upstream cascades that are required for virulence factor biosynthesis (Matsumoto *et al.,* 2021).

Manasa *et al.* (2015) examined the fungal endophyte diversity of *Suaeda filiformis*, a halophytic plant, growing in the Marakanam back waters of Tamil Nadu, India. *Fusarium chlamydosporum, F. solani, F. proliferatum, F. incarnatum, Aspergillus. flavus,* and *A. niger* were among the dominant fungal genera. Salinity tolerance of these fungal endophytes were evaluated under *in vitro* conditions, of the various isolates, *F. incarnatum* (MH593375.1) was found to salt tolerant growing at even 2M NaCl concentration. They showed that the endophyte was not only able to colonise salt-sensitive rice variety IR- 64, but also promoted seedling growth under salt stress and conferred salinity stress tolerance to its host. In this study, we demonstrated that the salt tolerant endophyte, *F. incarnatum* is able to vertically transmit itself to the next generation through seeds and in the next sporophytic generation was able to impart tolerance to salinity stress. We discuss these results in the context of using endophytes as a possible approach towards mitigating crop stress responses.

MATERIAL AND METHODS

Plant Material

Salt adapted endophyte *F. incarnatum* (Gen Bank Acc. No. MH593375.1) was evaluated for its ability to impart salinity tolerance to salt-sensitive rice variety IR 64. The seeds of IR 64 were surface-sterilized according to Arnold *et al.* (2000) and pre-germinated for 48 hours. Five-day-old *F. incarnatum* culture was used for inoculum preparation by washing the mycelial mat with sterile distilled water. Pre-germinated paddy seeds were treated with the mycelia suspension (2×10^6 cfu/ml) for 3 hours and another set of seeds treated with distilled water served as control or non-enriched seeds. Both *F. incarnatum* enriched and non-enriched pre-germinated IR 64 seeds were transferred to plastic pots (14 ×14 ×16 cm) and grown in a potting medium (red loam soil + farm yard manure). The experiment was carried out in three biological replications and two seedlings per pot were maintained. Seedlings were watered regularly and continued to grow till the maturity period. At the end of growing period, *F. incarnatum* enriched and non-enriched seeds were harvested and labelled as Trans Generation-1 (TG-1), E+ and E- seeds, where E+ refers to *F. incarnatum* enriched and E- refers to non-enriched seeds (Manasa *et al.,* 2015).

Seeds Obtained

F. incarnatum enriched and non-enriched TG 1 seeds obtained during the growing season 2017 were maintained at 4°C at School of Ecology and Conservation laboratory, University of Agricultural Sciences, GKVK, Bengaluru, Karnataka, India. Trans generation-1 (TG-1), E+ and E- seeds stored for two years were used to carry out present study.

Evaluation of Transgenerational Persistence of Salt Stress Tolerance in 7-day Old Paddy Seedlings

F. incarnatum enriched and non-enriched TG 1, IR 64 seeds were surface-sterilized according to Arnold *et al.* (2000) and germinated for 48 hours. Pre-germinated seeds were then transferred to moistened paper towels. One set of the paper towels were treated with 150mM NaCl and incubated at 28 ± 2°C for 7 days. Another set of seedlings were treated with sterile distilled water and maintained as control. Seven-day old seedlings were harvested and seedling lengths were measured. The experiment was carried out in three biological replicates with each paper towel having 10 seedlings. The treatments were E+S–; E–S–; E+S+ and E–S+, where E+E– refers to presence/absence of endophyte and S+/S– refers to presence / absence of salinity stress.

To further evaluate the ability of *F. incarnatum* on the performance of the IR 64 plants, pot experiments were carried out in the greenhouse. *F. incarnatum* enriched and non-enriched (E+S– and E–) 7-day old TG 1 seedlings were transferred to plastic pots (14 ×14 ×16 cm) and grown in a potting medium (red loam soil + farm yard manure). The experiment was carried out in three biological replications. In each pot, 2 seedlings were transplanted and the seedlings were watered regularly and continued to grow under control conditions till the harvest period.

Quantification of Native Hydrogen Peroxide (H_2O_2)

The amount of native H_2O_2 was quantified in the flag leaf of old 50-day old E+S– and E–S–, TG 1 plants raised in the greenhouse without salinity stress (E+S– and E–S). Flag leaves were excised and were cut into 5 cm bits. Tissue bits were washed with distilled water and transferred to test tubes and immersed in DAB (3,3' -Diaminobenzidine) staining solution for the detection of H_2O_2. Test tubes were wrapped with aluminum foil and incubated for 12 hrs in dark. After 12 hrs of incubation, excess stain was drained off and chlorophyll was removed for proper visualization of the stain. This was done by immersing the tissue bits in absolute ethanol and heating in a boiling water-bath for 10 min (or more if necessary, with intermittent shaking). Tissue bits were transferred to paper towel saturated with 60 per cent glycerol. Photographs of the tissue bits were captured to assess the amount of H_2O_2 being generated which was visualized as reddish brown stain formed by the reaction of DAB with the endogenous H_2O_2 (Lekshmy *et al.,* 2021).

Evaluation of Transgenerational Persistence of *F. incarnatum* in 60-day Old TG 1 Seedlings

The colonization ability of *F. incarnatum* was determined in 60-day old E+S– and E–S–, TG 1 seedlings. Re-isolation of the *F. incarnatum* was examined in leaf tissues. The explants (1 cm long) were surface sterilized as described above and inoculated on PDA and then incubated at room temperature for 5 days.

Fungal colonies emerging out of the endophyte enriched (E+) and non-enriched (E–) leaf tissue segments were classified as Operational Taxonomic units (OTU's). Imprints of the sterilized tissue segments were maintained to ensure effectiveness of surface sterilization. Fungi that emerged from the cut ends of the tissue were sub-cultured onto fresh PDA plates to obtain pure cultures. The purified isolates were cultured on PDA slants and stored at 4°C. Voucher numbers were assigned to each of the isolates and deposited in the School of Ecology and Conservation Lab, University of Agricultural Sciences, GKVK, Bengaluru, India.

Evaluation of transgenerational persistence of *F. incarnatum* induced growth promotion in later stages of seedling growth

To further evaluate the ability of *F. incarnatum* towards plant growth promotion in TG 1, IR 64 seedlings, 7-day old E+S– and E–S–, TG 1 seedlings were maintained in the green house as described above. Phenotypic observation of plant biomass was recorded at 90 and 120-day old plants. At the end of growing season, endophyte enriched and non-enriched seeds were harvested and stored to carry out further experiments.

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) with SPSS version 20. Duncan's multiple-range test was used to evaluate the significance of differences between treatments.

RESULTS AND DISCUSSION

Endophyte enriched and non-enriched trans generation (TG 1) seeds of IR 64 variety were analyzed for seedling performance on paper towels under 150mM NaCl stress. *F. incarnatum* fungi induced a significant increase of growth in 7-day-old seedlings under salinity stress, when compared to seedlings which were grown without the endophyte (Fig. 1). The growth-promoting effect was not detectable for seedlings, which were grown without salt stress (Fig. 2).

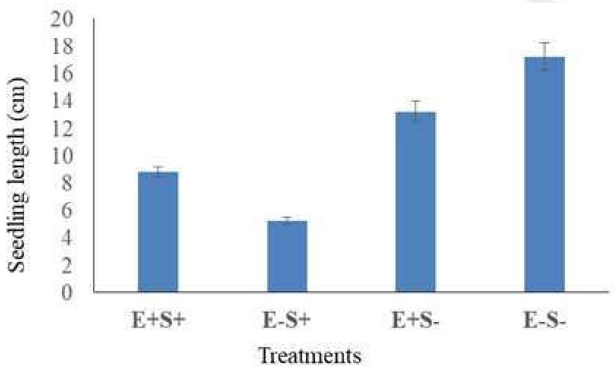

Fig. 1:Seedling growth of 7-day old TG 1 seedlings under different treatments (E+S+; E-S+; E+S-; E-S- where E+/ E-: Endophyte enriched/non-enriched and S+/S-: Presence/absence of salinity stress (150 mM NaCl). (N=30 number of seedlings). Line on bars indicates standard error over mean (±)

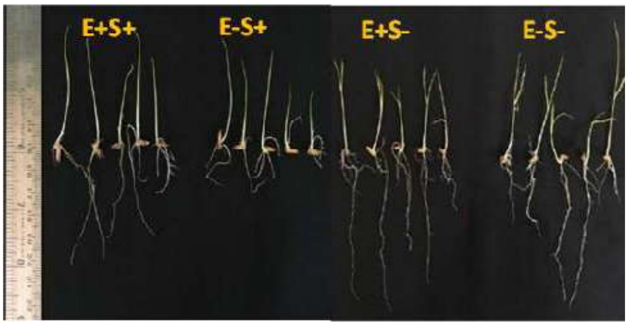

Fig. 2: Phenotypic growth of 7-day old TG 1 seedlings as influenced by endophyte colonization under salt stress. E+/E-: Endophyte enriched/non-enriched and S+/S-: Presence/absence of salinity stress (150 mM NaCl)

Reactive Oxygen Species (ROS) generation is a common response to stress in plants, particularly in chloroplasts and mitochondria and are the major sites for oxidative respiration. H_2O_2 localization was used as a proxy to assess accumulation of ROS (Azad and Kaminskyj, 2016). To localize the H_2O_2, endophyte enriched and non-enriched 50-day old plants grown under control conditions (without salinity stress) were tested by treating flag leaves with DAB stain. The H_2O_2-DAB reaction formed a brown pigment in the leaf tissue. The study showed that endophyte enriched plants had noticeably less brown pigmentation than non-enriched plants, suggesting lower ROS level (Fig. 3). These results suggest that besides promotion of basic processes associated with growth and development, the *F. incarnatum* reduces the cell membrane damage by reducing the amount of H_2O_2 production.

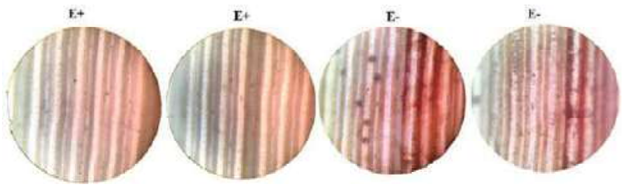

Fig. 3: Histochemical staining of hydrogen peroxide (H_2O_2) by DAB (3,3' -Diaminobenzidine) stain in 50-day old endophyte enriched (E+) and non-enriched (E-) control (without salinity stress) TG 1 leaves

In order to demonstrate the trans-generational persistence of *F. incarnatum* in TG 1seedlings, colonization ability of *F. incarnatum* was determined in 60-day old TG 1 plants. Intriguingly, non-enriched 60-day old TG 1 leaf tissues were completely devoid of fungal colony, however there was one particular fungal OTU emerging in endophyte enriched E+tissues (Fig. 4). Based on the morphological indices like colony colour, mycelia growth and in comparison with the pure culture of *F. incarnatum* it was confirmed that endophyte enriched TG 1 leaves were colonized by *F. incarnatum* in trans-generation seedlings.

Fig. 4: Re isolation of *F. incarnatum* in 60-day old endophyte enriched (E+) leaves. Note that there was no emergence of the fungus from the non-enriched leaves (E-)

When *F. incarnatum* enriched and non-enriched control TG 1 IR 64 plants were maintained in the greenhouse, *F. incarnatum* induced a significant increase in shoot growth of 90 day-old plants

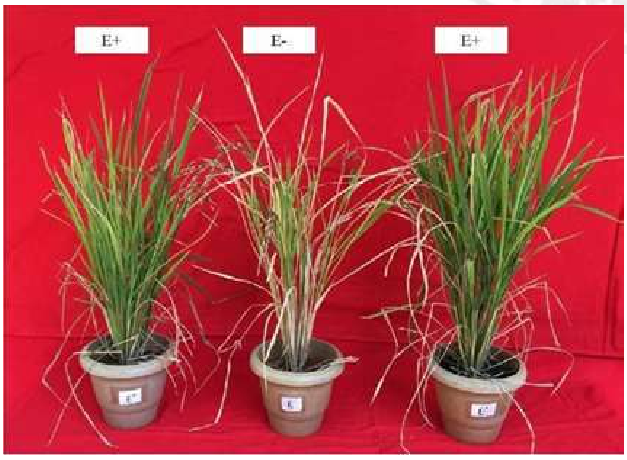

Fig. 5: Phenotypic growth of 90-day old TG 1, IR 64 plants as influenced by *F. incarnatum* colonization. E+ represent the endophyte enriched and E- represents the non-enriched control (without salinity stress) plants

(Fig. 5). *F. incarnatum* was also able to improve overall seedling biomass including shoot and root growth in 120 day old plants (Fig. 6).

Fig. 6: Phenotypic growth of 120-day old TG 1 IR 64 plants as influenced by *F. incarnatum* colonization. E+ represent the endophyte enriched and E- represents the non-enriched control (without salinity stress) plants

The study demonstrated that a salt tolerant endophyte *F. incarnatum*, is vertically transmitted through seeds to the next generation in rice and also results in an enhanced protection of plants against salt stress in subsequent sporophytic generation. Work is underway to address the molecular mechanism by which *F. incarnatum,* imparts salinity stress tolerance across generations. The results of the study open up avenues to explore the use of endophytes to manipulate crop responses to stress and perhaps help crop plants to cope up in stressful environments.

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