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Fungal Endophytes Enhance Salinity Stress Tolerance in Tomato (*Solanum lycopersicum* L.) Seedlings

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Abstract

Endophytes are microorganisms such as bacteria or fungi that reside inside the plant tissues without causing any apparent diseases to the host. This symbiotic association is known to improve host fitness and enhance its tolerance to adverse environmental conditions. As an eco-friendly way to enhance crop adaptation to stress, there is a growing interest in identifying candidate endophytes. The present study analyzed the stress tolerance potential of ten endophytes isolated from different plants inhabiting different locations across India. Four endophytes (PJ-9, SF-5, K-23 and V4-J) sustained their growth even at 2.5 M NaCl, indicating superior stress tolerance potential. These endophytes were used to inoculate a tomato variety (*Arka Surabh*) which is sensitive to salt stress. The results demonstrated that endophytic fungi can successfully colonize in salt-sensitive tomato variety and impart salinity stress tolerance. Amongst the ten selected endophytes, SF-5 showed significantly higher seedling growth (9.99 ± 0.27 cm) when compared to control (7.41 ± 0.16 cm). Conclusively, the experimental results indicated the potential role of endophytes in interacting with new host plants for enhancing growth and tolerance to salinity stress.

Keywords : Endophytes, Tomato, Salinity, Seedling vigour, Plant tolerance

Globally, soil salinity is one of the important concerns in agriculture. Localized accumulation of soluble salts in soil impairs plant growth and development, ultimately hampering crop yields. As per recent estimates, 20 per cent of the global cultivated land is saline, including 33 per cent of the irrigated agricultural land. In the current trend, a 33 per cent increase in soil salinization is expected by 2050 (Otlewska *et al*., 2020). The majority of the crop plants are evolved and cultivated on arable land as glycophytes and are sensitive to salt stress (Panta *et al*., 2014 and Zorb *et al*., 2019), exhibiting an average reduction of 50 - 80 per cent in yields under moderately saline conditions of 4 - 8 dS/m (Panta *et al*., 2014). Here, the penalty on yield depends upon the crop-specific threshold of salinity tolerance (Zorb *et al*., 2019).

Endophytes, bacteria or fungi, inhabit the inter cellular spaces of plant tissues and feed on apoplastic nutrients (Shahzad *et al*., 2018 and Vardharajula *et al*., 2017). The endophytes play a significant role in equipping the plants to tolerate diverse adverse environmental conditions (Sampangi-Ramaiah *et al*., 2020 and Manasa *et al*., 2020). This symbiotic association often confer a wide array of benefits to the host including improved growth, tolerance to biotic and abiotic stresses and improved nutrient acquisition (Chitnis *et al*., 2020). Hence, harnessing the benefits of the symbiotic association is considered a promising eco-friendly way of enhancing abiotic stress tolerance in susceptible crops (Fan *et al*., 2020). It is also demonstrated that habitat adapted endophytes from extreme environmental conditions such as drought, salinity, heat, etc. are capable of imparting stress tolerance in non-host crop plants (Rodriguez *et al*., 2008; Sangamesh *et al*., 2018 and Ripa *et al.,* 2019). Plants respond to salt stress through multiple mechanisms operating at cellular, molecular and biochemical modifications to minimize stress effects (Bahmani *et al*., 2015 and Otlewska *et al*., 2020). Endophytes are capable of triggering several of these stress adaptive mechanisms in sensitive crops while sustaining crop yields (Sampangi-Ramaiah *et al*., 2020 and Gupta *et al*., 2021). Hence, it would be useful to identify salt-tolerant endophytic strains and to validate their capability to impart stress tolerance in economically important sensitive crops.

The present study analysed the stress tolerance nature of 10 habitat-adapted fungal endophytes collected from different locations in India and tested their ability to colonize and confer salinity tolerance in a comparatively sensitive tomato variety. Tomato is the second most-consumed vegetable crop after potato and is known to be sensitive to soil salinity at multiple crop growth stages (Foolad *et al*., 2007). We demonstrate that selected endophytes enhance salinity tolerance in tomato variety, *Arka Saurabh*.

Material and Methods

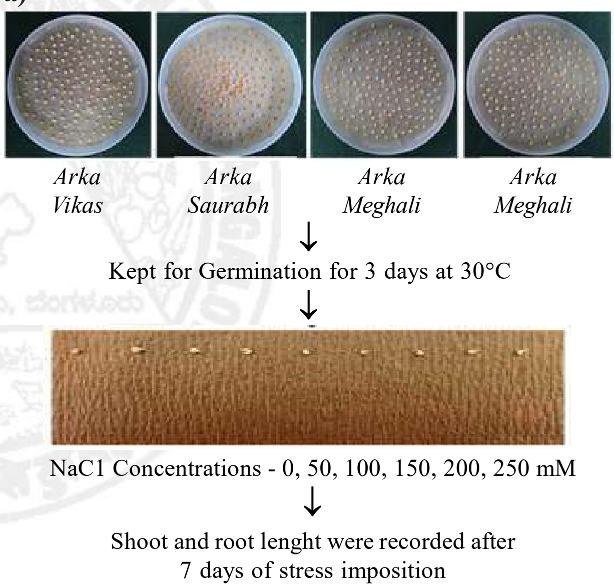
Collection of Fungal Endophytes, Maintenance, and *in-vitro* Screening for Salinity Stress Tolerance

Ten fungal endophytes were collected from the School of Ecology and Conservation Laboratory, Department of Crop Physiology, University of Agricultural Sciences, Bangalore, India. These isolates were collected from different locations in India from wild plants and designated as K-23 and K-26 (collected from Kargil - J&K, 34°34'22"N, 76°7'57"°E), P-10 and P-37 (Pangong Tso, 33°43'2.74"°N, 78°53'29.08"°E), SF-5 (Tamil Nadu, 11.1271°N, 78.6569°E), PJ-9 (Karnataka, 15.3173°N, 75.7139°E), V4J (Kerala, 9.9667°N, 76.3168°E), LAS-6 (Thar desert, 27.4695°N, 70.6217°E) and cultures 1082 and 1083 obtained from the collections of Dr. T. S. Suryanarayanan, Vivekananda Institute of Tropical Mycology (VINSTROM), Chennai. These isolates were sub-cultured on Potato Dextrose Agar (PDA) medium, using pure culture slants and maintained at 4ºC for further use. Screening of the different endophytes for salt stress tolerance was performed on PDA media supplemented with different concentrations (0, 0.5, 1, 1.5, 2 and 2.5M) of sodium chloride (NaCl) along with respective controls. In each case, five-day-old colony culture with actively growing mycelial disc measuring around 0.9 cm in diameter was placed at the center of the PDA plates (90 mm, Tarsons, India). After five days of post-inoculation, salinity tolerant fungal endophytes were selected for further studies based on the measurement of colony diameter.

Determination of Lethal Concentration of NaCl in Tomato Seedlings

The lethal concentration of salt stress for tomato seedlings was determined by treating different concentrations of NaCl on four popular varieties of tomato namely, *Arka Vikas*, *Arka Saurabh, Arka Meghali* and *Arka Abha*, procured from Indian Institute of Horticultural Research, Hessaraghatta, Bengaluru, India. The seeds were initially surface

a)



b)

Lethal Concentration (LC_{50})

Varieties	Seedling growth		
	UB	UB	LC50
Arka Vikas	151.86	129.93	141.07
Arka Saurabh	143.41	120.59	132.34
Arka Meghali	162.49	140.28	151.37
Arka Abha	148.66	123.56	136.43

Fig. 1: Determination of LC_{50} value in tomato seedlings under salt stress a) Methodology followed to induce salinity stress; b) Determination of LC50 value using SPSS software and probit analysis (UB- Upper Bound, LB-Lower Bound).

sterilized using 4 per cent (v/v) sodium hypochlorite (NaOCl) for 3 min, followed by 70 per cent (v/v) ethanol treatment for 30s and then rinsed with autoclaved double distilled water. The surface-sterilized seeds were then germinated on moist blotting paper in an incubator maintained at 28ºC. Later, uniformly germinated seeds were selected and placed on a germination paper amended with varying concentrations of NaCl (0, 50, 100, 150, 200 and 250 mM) (Fig. 1a). Root length and shoot lengths were recorded on seven days after stress imposition (Rashed *et al.*, 2016) and LC_{50} value was determined using the statistical software IBM SPSS statistics 20 (https://www.ibm.com/in-en/analytics/spss-statistics-software).

Screening of Endophytes for Salinity Stress Tolerance in Tomato

The selected salinity tolerant endophytes were tested for their ability to impart salinity tolerance in tomato. For this, an initial inoculum at a mycelial dose of $2x10^6$ CFU (colony forming units) was used and pre-germinated uniform seeds of tomato were surface sterilized and suspended in inoculation media for three hours (Zhang *et al.*, 2014 and Manasa *et al.*, 2020) for colonization. Further, the seeds were rinsed with autoclaved distilled water to remove traces of mycelia attached and subjected to salinity stress conditions at the selected LC_{50} concentration of NaCl and ddH_2O as control and incubated at 27ºC. The root and shoot length were recorded seven days of post-incubation to analyze the effect of endophyte treatment on tomato seedlings. Three replicates were maintained for Analysis of Variance (ANOVA) and Duncan's multiple range test (DMRT) was followed to compare between treatment means of the experiment.

Re-Isolation and Characterization of Fungal Endophytes

The fungal endophytes were further re-isolated from the treated tomato stem and root tissues to confirm the endophyte colonization and characterization. For this, initially, the tissues were cut into 1 cm long segments and surface sterilized with 70 per cent (v/v) ethanol for 50s followed by sequential washes in 1 per cent (w/v) NaOCl for 1min, 70 per cent (v/v) ethanol for 30s and finally rinsed in sterile distilled water for thrice and blot dried under aseptic conditions (Arnold *et al.*, 2000). Imprints of sterilized cut segments were taken on PDA plates to ensure the effectiveness of surface sterilization (Schulz *et al.*, 1993). The surface-sterilized plant segments were placed on petri dishes containing PDA and incubated for five days (Suryanarayanan, 1992) for endophytic fungal emergence. The fungal growth from the cut ends of segments was transferred to fresh PDA plates using a sterile needle to obtain pure cultures. The pure cultures were then compared with respective mother cultures for their morphology *viz*., colony appearance, conidia and hyphae using a microscope (EVOS™ M7000 microscope, UK) (Domsch *et al.*, 1980 and Arx, 1981). Genomic DNA of fungal mycelium was isolated following the cetyltrimethyl ammonium bromide (CTAB) method (Rogers and Bendich, 1994) and polymerase chain reaction (PCR) was carried out to amplify the ITS region of genomic DNA using ITS1 and ITS4 (White *et al.*, 1990) as forward and reverse primers respectively. The 600 bp PCR product amplified was purified and sequenced. The FASTA sequence was BLASTn searched in NCBI GenBank database (*www.ncbi.nlm.nih.gov*). Based on the maximum homology and per cent similarity, identity is assigned to endophytes using the criterion described by Higgins *et al.* (2007). The phylogenetic analysis was carried out using Clustal W plugin from MEGA software, version 7.0 (Kumar *et al.,* 2016). Phylogenetic relatedness was determined by employing a neighbor-joining method (Saitou and Nei, 1987) with 1000 bootstrap replications (Felsenstein, 1985).

Results and Discussion

Evaluation of the Salinity Tolerance Level of Selected Endophytes and Selection of Promising Candidates

Initially, the selected endophytes were screened for their ability to tolerate salt stress of varying

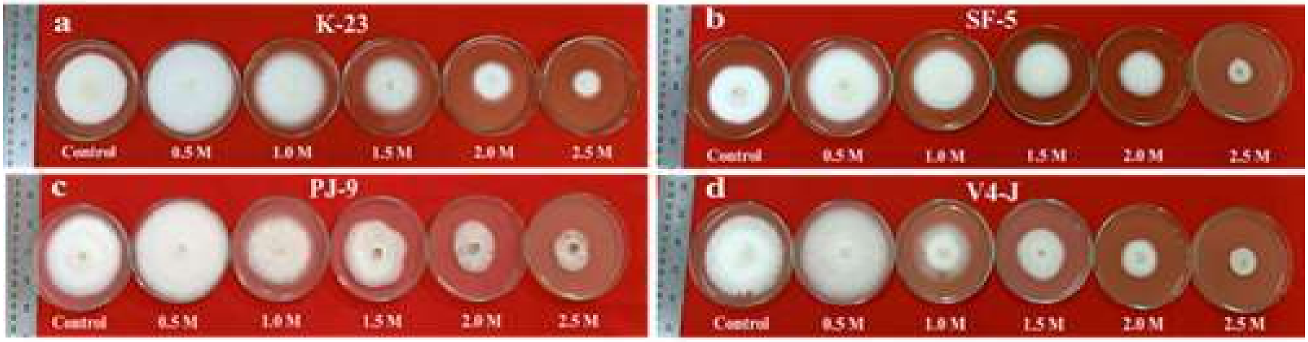


Fig. 2: Representative image showing the growth of fungal endophytes amended with different concentrations of NaCl (0, 0.5, 1, 1.5, 2 and 2.5M) on potato dextrose agar (PDA) media after 5 days of inoculation a) K-23; b) SF5; c) PJ-9; d) V4-J

concentrations. All the endophytes except 1083 sustained growth up to 1M NaCl, indicating their tolerance to stress (Fig. 2a). While all the nine endophytes survived up to 1.0M NaCl, the isolates K-23, SF-5, PJ-9 and V4-J exhibited good growth up to 2.50 M NaCl (Fig. 2a-d). In previous studies, it has been shown that endophytic fungi isolated from naturally growing plants in harsh environments can tolerate extreme stresses (Ruppel *et al*., 2013, Azad and Kaminskyj, 2016; Sangamesh *et al*., 2018; and Sampangi-Ramaiah *et al*., 2020). Fungal endophytes isolated from halophytic plants inhabiting desert areas tolerate up to 3.5- 4M NaCl stress (Jalili *et al*., 2020). Several studies demonstrate that such habitat adapted endophytes boost growth, promote nutrient uptake and enhance stress tolerance in crop plants (Ikram *et al*., 2018; Soldan *et al*., 2019).

Evaluation of the Salt Stress Tolerance in Tomato and Identification of LC_{50}

In domesticated tomato, the selection for large fruit size has resulted in reduced salt tolerance (Xiang and Jimenez-Gomez, 2020). Most cultivated varieties exhibit variation in their tolerance to salinity stress (Alam *et al*., 2021). Fig. 1a is a schematic representation of the workflow. LC_{50} values observed for *Arka Vikas, Arka Meghali* and *Arka Abha* were 141.07, 151.37, and 136.43mM, respectively, (Fig. 1b). Among four different tomato varieties used in the present study, *Arka Saurabh* exhibited the highest susceptibility to salt stress with an LC_{50} of 132.34mM, when compared with others. Fig. 3 indicates the effect of salt stress on the growth of the seedlings of *Arka Saurabh*.

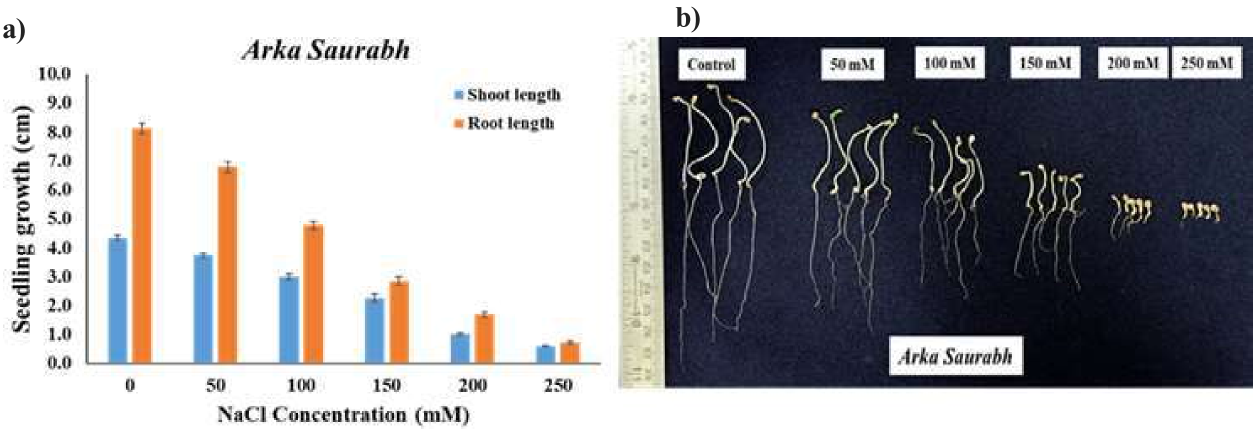


Fig. 3 : Effect of salinity stress on the growth of tomato seedlings (*Arka Saurabh*). a) Effect of different concentrations of salt (0, 50, 100, 150, 200, 250mM) on shoot and root length (cm); b) Representative image of phenotype of the seedlings on treatment with different concentrations of salt on the seedling growth of tomato

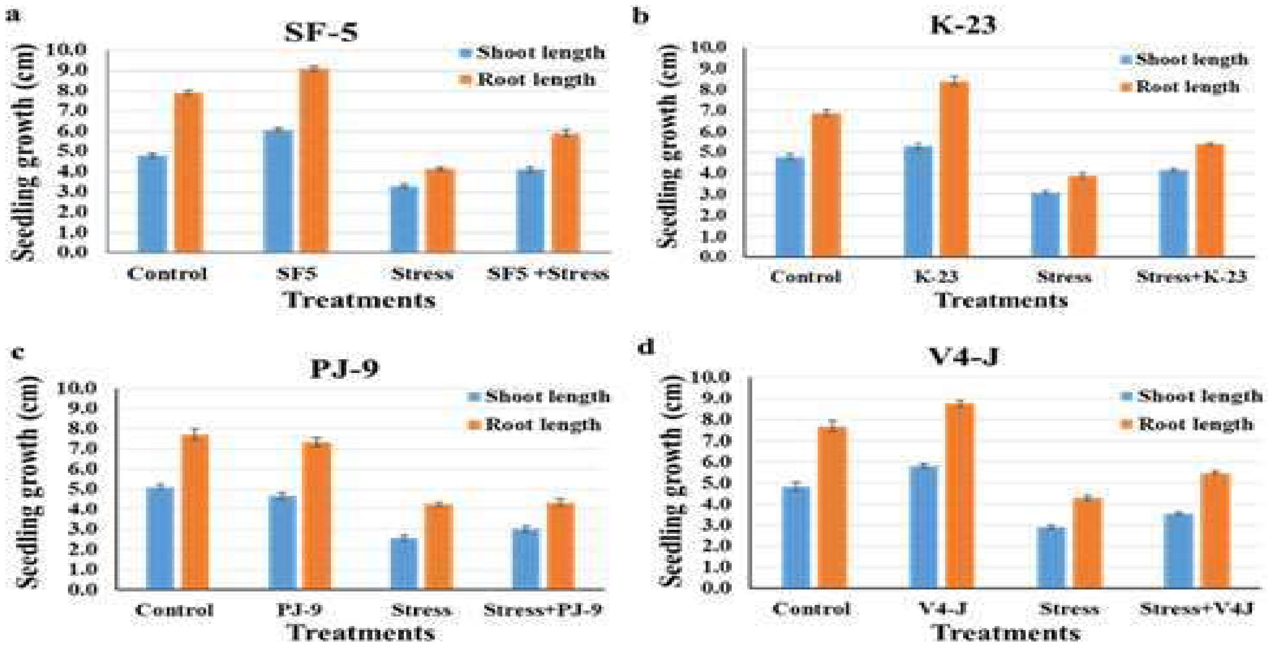


Fig. 4: Effect of endophyte treatment on seedling growth of tomato (*Arka Saurabh*) under control and 132.34mM NaCl stress. Effect of the treatments on root length and shoot length of a) SF-5; b) K-23; c) PJ-9; d) V4-J (data represents an average of *n* = 30 seedlings)

Endophytes Improve Salinity Stress Tolerance in Tomato

Several studies on plant growth-promoting endophytes have shown to impart salt stress tolerance in a range of plants including wheat, rice, ryegrass, *Arabidopsis* and poplar (Jan *et al*., 2019; Kasotia *et al*., 2015 and Abdelaziz *et al*., 2017). From recent studies, it has been observed that 60 per cent of fungal endophytes isolated from the plants of the harsh environment can improve host plant tolerance under laboratory conditions (Zhou *et al*., 2015; Sangamesh *et al.,* 2018; Manasa *et al*., 2020 and Sampangi-Ramaiah *et al*., 2020). In the present study, inoculation of fungal endophyte suspensions to the seeds of a salt-sensitive tomato variety (*Arka Saurabh*) significantly improved seedling growth under salt stress, when compared to uninoculated control tomato seeds (Fig. 4a-d). Among the four potent fungal endophytes, SF5 increased seedling growth (9.99 ± 0.27 cm) significantly compared to other isolates and control (7.41 ± 0.16 cm) under salinity stress conditions (Fig. 4a). To confirm the colonization of the fungal endophytes in tomato, the endophytes were re-isolated and compared with the mother culture for their morphology (colony appearance, conidia and hyphae). The re-isolated endophytes were also subjected to molecular characterization using ITS 1 and ITS 4 primers. The sequence data of 600bp PCR product confirmed the identity and *Fusarium* species was the closest match based on phylogeny (Fig. 5). This suggests that salt-tolerant endophytes could successfully colonize the salt-sensitive tomato variety *Arka Saurabh* and confer salt stress tolerance. Though the precise mechanisms through which such effects are manifested are not clear, this study opened up the possibility of exploring these fungal resources in modulating the growth of crops under stressful environments. It is clear that endophytes can

Fig. 5: Re-isolation and confirmation of the fungal colonization in tomato seedlings. Representative image of a) re-isolated endophyte; b) mother culture; c) conidia (40X); d) PCR-amplification using ITS primers

effectively modulate plant responses to abiotic stresses and help in mitigating the adverse consequences of abiotic stress (Sampangi-Ramaiah *et al*., 2020).

Exploiting the ability of endophytes to confer stress tolerance to plants and using them in stress mitigation could be a novel and durable strategy to sustain crop production under changing climatic conditions. This study demonstrated the importance of prospecting endophytes from extreme habitats to improve salinity stress tolerance in tomato. The select endophytes can also provide excellent models for understanding stress tolerance mechanisms that can be targeted in crop plants. In summary, besides other conventional approaches, the use of endophytes could help accelerate efforts towards developing crop plants resilient to climate change.

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