Endophytic fungi of salt adapted *Ipomea pes-caprae* L. R. Br: their possible role in inducing salinity tolerance in paddy (*Oryza sativa* L.)

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Endophytic fungi occur ubiquitously in all plants. Though their origin and evolution is enigmatic, they are known to play an important role in plant growth and development. Here we explore the endophytic fungal diversity of a perennial creeping vine, *Ipomea pes-caprae* (family Convolulaceae), occurring naturally in the coastal sand dunes of peninsular India. Of the ten endophytes isolated from the plant, *Fusarium oxysporum* (MH511104) was found to grow even at 2 M NaCl in potato dextrose agar medium. The fungus was able to successfully colonize and impart salinity tolerance to salt-sensitive paddy variety, IR-64. We discuss these results in the context of increasing global interest on endophytes as a possible alternative route to crop improvement.

Keywords: Endophytic fungi, *Ipomea pes-caprae*, paddy, salt stress.

IPOMEA PES-CAPRAE (L.) R.Br. (Convolulaceae), commonly known as morning glory, is the predominant flora of coastal sand dunes of the tropics[1]. The plant extracts exhibit anti-inflammatory[2], anti-microbial[3] and anti-cancer[4] activities and are also a rich source of antioxidants, phenols and flavonoids[5]. Little is known about the endophytic fungal diversity of *I. pes-caprae* and its ability to tolerate salinity stress. Interest on endophytes of plants adapted to extreme habitats such as cold and hot desert and saline habitats has been gaining attention following Rodriguez *et al.*[6] who showed that such endophytes could also transfer their stress tolerance trait to non-host plants like crops.

Soil salinity is widely regarded as one of the most important abiotic stresses affecting about 20% of global arable land area[7]. Saline soils affect plant growth and development leading to reduced productivity[8]. Paddy, the major cereal crop of India, is sensitive to salinity stress at all growth stages, especially at early vegetative stage[9].

We studied the endophytic fungal diversity of *I. pes-caprae* and evaluated its ability to tolerate salinity stress. We also examined the role, if any, of such endophytes in imparting salinity stress tolerance to salt-sensitive paddy variety, IR-64. We discuss the results in the larger context of the utility of endophytic fungi as a tool in improving crop plant adaptability to abiotic stresses.

I. pes-caprae plants growing naturally in the shore line of Marakkanam, Villupuram, Tamil Nadu, India (12°11′N, 79°55′E) and free of any disease symptoms were randomly selected and uprooted carefully using a trowel and placed in brown paper bags, labelled and brought to the laboratory. Voucher herbarium specimen were prepared and deposited at the School of Ecology and Conservation Laboratory (SEC Lab), University of Agricultural Sciences, GKVK, Bengaluru, India.

Leaf, stem and root of *I. pes-caprae* were washed in running tap water, cut into segments and surface sterilized according to Arnold *et al.*[10]. Sterilized tissue segments (1 cm × 1 cm) were placed aseptically on water agar (2%) and incubated (26°C under 12 h of light/dark cycles) until fungal emergence. The effectiveness of surface sterilization was confirmed by the absence of any fungal growth in the imprints of the tissue following Schulz *et al.*[11]. Three replicates, each with five segments, were maintained separately for different tissues. Fungal mycelia emerging from the cut end of tissues were purified onto potato dextrose agar (PDA) plates, incubated as described above and stored at 8°C. Purified isolates were separated into operational taxonomic units (OTUs) based on colony characteristics like colour, growth pattern, spore and conidia morphology[12–14]. Voucher numbers were assigned to all fungal OTUs, documented and deposited at culture collection maintained at SEC lab, for further studies.

Colonization frequency of the endophytes was calculated according to Huang *et al.*[15]. Diversity indices such as species richness, Shannon index and Simpson index were calculated using Past.exe (version 2.1)[16].

Molecular identification of the endophytic fungal OTUs was done by amplifying the 28s ribosomal region of the internal transcribed spacer (ITS). The 100 mg of five-day-old endophytic fungal mycelium was used for DNA isolation according to Doyle and Doyle[17]. The polymerase chain reaction (PCR) was carried out with a DNA concentration (30 µg/ml) to amplify the ITS region of genomic DNA using ITS1 and ITS4 as forward and reverse primers respectively[18]. The PCR product amplified at 600 bp was sequenced (Shrimpex Biotech Services Pvt Ltd, Chennai, India). The nucleotide sequences were edited using DNA editing software, MEGA 7 and FASTA sequences were used as a query in BLASTn in NCBI GenBank (www.ncbi.nlm.nih.gov) to identify the fungal genera or species using the criterion described by Higgins *et al.*[19]. The phylogenetic relatedness of the fungal ITS sequences was determined by employing the neighbor-joining (NJ) method[20]. Sequences were aligned using ClustalW and incorporated into MEGA 7 software, version 7.0 for NJ phylogeny[21]. The statistical support for the nodes was tested on the basis of 1000 bootstrap generated datasets[22].

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Table 1. Percentage colonization, number of fungal isolates, species richness and species diversity indices of endophytic fungi in leaf, stem and root of *I. pes-caprae*

Plant part	Colonization (%)	No. of fungal isolates	Species richness	Simpson's diversity index (D')	Shannon's diversity index (H')
Leaf	13.33	7	4	0.84	1.86
Stem	100	15	7	0.78	1.55
Root	26.67	5 2	0.67	0.48	

Five-day-old colony cultures (8 mm diameter) were inoculated aseptically in sterile petri plates (90 mm, Tarson, India) containing PDA amended with 1 M NaCl along with respective control and incubated as described above for five days. The growth of fungi was recorded by measuring the colony diameter after five days of inoculation. For sporulating fungi, two mycelial plugs (8 mm dia) were placed into 100 ml potato dextrose broth (PDB) amended with 1 M NaCl. Flasks were incubated in shakers with 120 rpm/min for 5 days at 26°C. Mycelial mats were harvested, dried and weighed. Endophytes tolerant to 1 M NaCl were further screened for tolerance up to 3 M NaCl on PDA.

Oryza sativa seeds, variety-IR-64 (procured from the Department of Crop Physiology, UAS, GKVK, Bengaluru) were surface sterilized using 4% NaOCl v/v (3 min) followed by 70% ethanol (v/v) (30 s) and washed with autoclaved double distilled water. The 48 h old seedlings were treated with endophytic fungi according to Zhang *et al.*[23] with minor modifications. *Fusarium oxysporum* (MH511104) which was found to be salt tolerant, was grown on PDA for five days, harvested and prepared as the inoculum at a mycelial concentration of 2×10^6 CFU (colony forming units). One set of seedlings was soaked completely (root and shoot) with above fungal inoculum (+EF) and another with water (–EF) for 4 h. Further, seedlings were repeatedly washed with autoclaved distilled water to remove traces of mycelia attached to seedlings. The +EF and –EF seedlings were further subjected to treatments of 200 mM NaCl and control (water) for 5 days. Sixty seedlings were maintained for each treatment and root and shoot length was recorded after five days of treatment.

All experiments had a minimum of three replications. Analysis of variance (ANOVA) was used wherever required to statistically validate the results obtained. Duncan's multiple range test (DMRT) was used to compare the means. All analysis were conducted using PAST.exe (version 2.14)[16].

Twenty seven endophytic fungal isolates were obtained from the various tissue explants of *I. pes-caprae*. The highest colonization by the fungi was observed in stem segments (100%) followed by 26.67% in root and 13.33% in leaf; the species diversity of the endophytes was higher in leaves than that in the stem or root (Table 1). Species richness was highest in stem followed by in leaf and root. On the basis of morphological traits including colour, growth pattern, spores and conidia morphology, the 27 isolates were assigned to 10 OTUs. Molecular characterization of the 10 OTUs using universal ITS primers (ITS1 and ITS4) showed that the fungi were *Alternaria alternata*, *Aspergillus flavus*, *Cladosporium oxysporum*, *C. tenuissimum*, *Dioporthe phaseolorum*, *Fusarium chlamydosporum*, *F. oxysporum*, *F. proliferatum*, *Lasiodiplodia pseudotheobromae* and *Penicillium spinulosum* (Supplementary Table 1). The NCBI GenBank accession numbers of these fungi are MH511101 to MH511109 and MK729535 (Supplementary Table 1). NJ phylogenetic consensus tree inferred from the rDNA ITS sequences of the fungal OTUs is presented in Figure 1. The figure shows the closest match along with their respective GenBank accession numbers. The fungi, *A. alternata* (MH511108) and *C. tenuissimum* (MH511109) were most abundant (22%) whereas *C. oxysporum* (MH511102), *F. oxysporum* (MH511104) and *F. proliferatum* (MH511106) were least abundant (~4%) (Figure 2).

All the 10 fungal isolates were evaluated for their ability to tolerate salinity stress. As seen from Figure 3, except *D. phaseolorum* (MH511101), *F. oxysporum* (MH511104) and *F. chlamydosporum* (MH511105), the mycelial growth was strongly inhibited at 1 M NaCl. The change in colony growth at 1 M NaCl over control was 20% (in *C. tenuissimum*, MH511109) compared to 88% (in *L. pseudotheobromae*, MK729535). Whereas in *D. phaseolorum* (MH511101) and *C. oxysporum* (MH511102) there was no difference in growth due to stress. In *F. oxysporum* (MH511104), there was a 54% increase in growth in the presence of salt (Figure 3). In media amended with a graded concentration of NaCl, it grew maximally at 0.5 M NaCl and retained the ability to grow even at 2 M NaCl (approximately 11.7% salinity stress) (Figure 4). Thus, it appears that *F. oxysporum* (MH511104) could be a halophilic fungal isolate adapted to the coastal and saline habitats in which its host plant, *I. pes-caprae*, is naturally distributed.

An attempt was made to study if the halophilic isolate, *F. oxysporum* (MH511104) is able to colonize salt-sensitive paddy genotype, IR-64 and if so, whether the fungus is also able to impart salinity tolerance to the crop. Inoculation of the salt-sensitive IR-64 paddy seedlings with *F. oxysporum* (MH511104), significantly improved

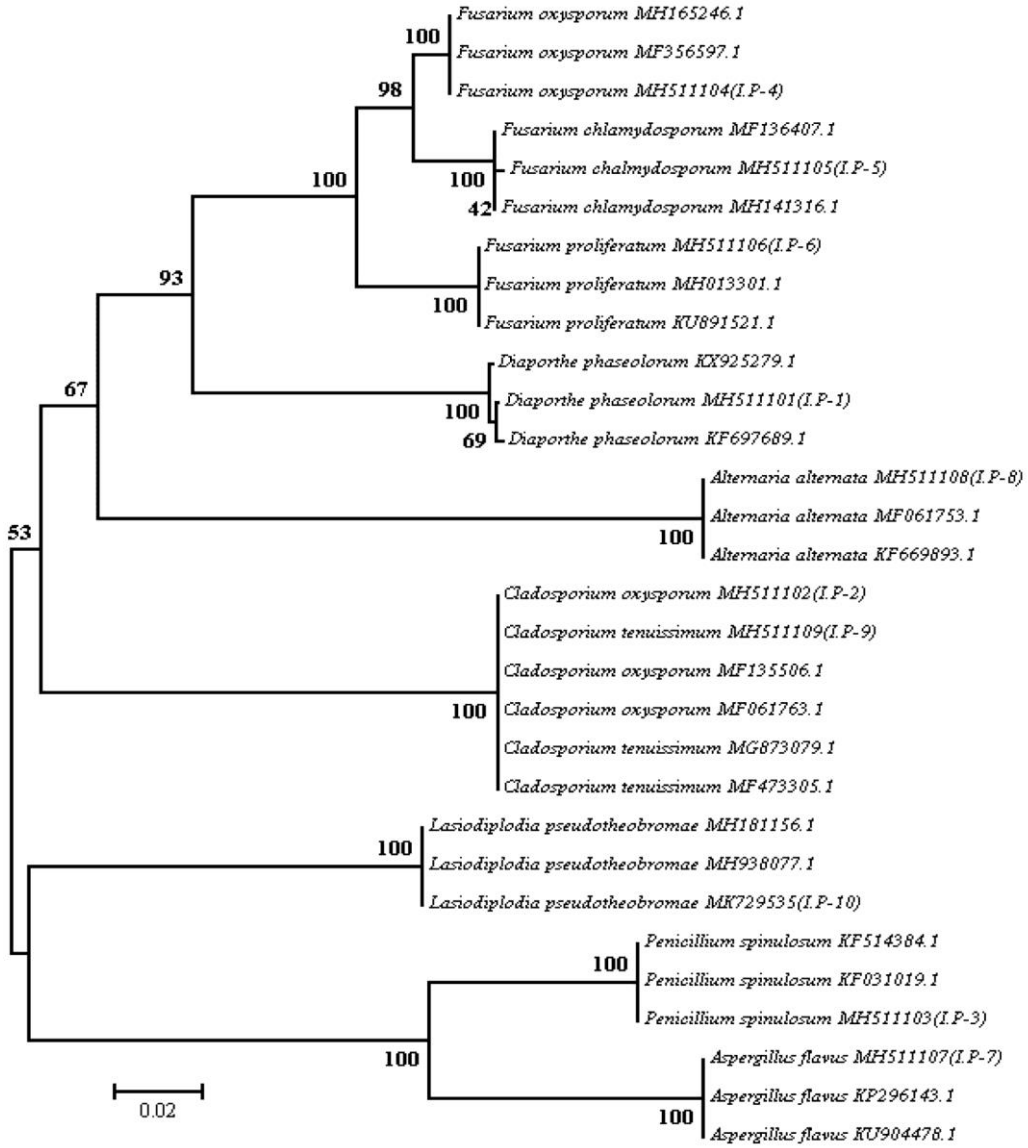

Figure 1. Neighbor-joining phylogenetic consensus tree inferred from rDNA ITS sequences of endophytic fungal OTUs obtained from salt adapted *I. pes-caprae*. The tree was constructed with bootstrap value of 1000 replicates. Fungi suffixed with the letter I.P. are from this study.

seedling growth under salt stress compared to seedlings not treated with endophyte (Figure 5 *a*, *b*). Seedling growth upon fungal infection, however, remained unaltered under controlled conditions (Figure 5 *a*). The endophyte treatment increased root and shoot growth by 46% and 50% respectively, under salt stress compared to un-inoculated seedlings (Figure 5 *a*).

Endophytic fungi dwell in the intercellular spaces of all plants[10]. Diverse fungal assemblages from plants growing in saline soils and salt marshes have been reported[24]. However, limited information is available on salt tolerance levels of endophytes of saline habitat-adapted plants[26–28] and their role in imparting salt tolerance to non-host plants[6,29]. The endophyte species diversity recovered from *I. pes-caprae* reflects that reported earlier from several halophytes; most of the fungi belong to Ascomycetes group[1,24,27,30]. In fact, several spore producing, dark septate endophyte genera like *Alternaria*, *Aspergillus*, *Cladosporium*, *Penicillium* recovered from *I. pes-caprae* are common associates of saline habitat plants[30]. In the present study, the genus *Fusarium* was found to be dominant. It is interesting to note that Beena *et al.*[1] also found the same genus to be the dominant fungus in roots of *I. pes-caprae*. Though it is not immediately clear if such associations are phylogenetically shaped, it would be of interest to examine their functional relevance, especially if they help to modulate plant responses to environmental stress.

Among the 10 fungal isolates recovered from *I. pes-caprae*, *F. oxysporum* (MH511104) was noteworthy for

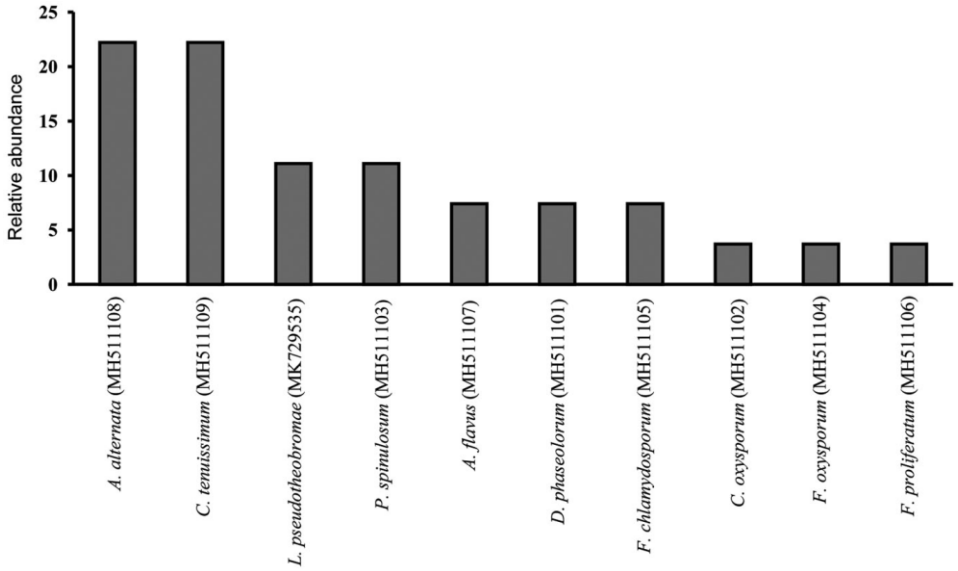

Figure 2. Relative abundance of endophytic fungi isolated from *I. pes-capre*.

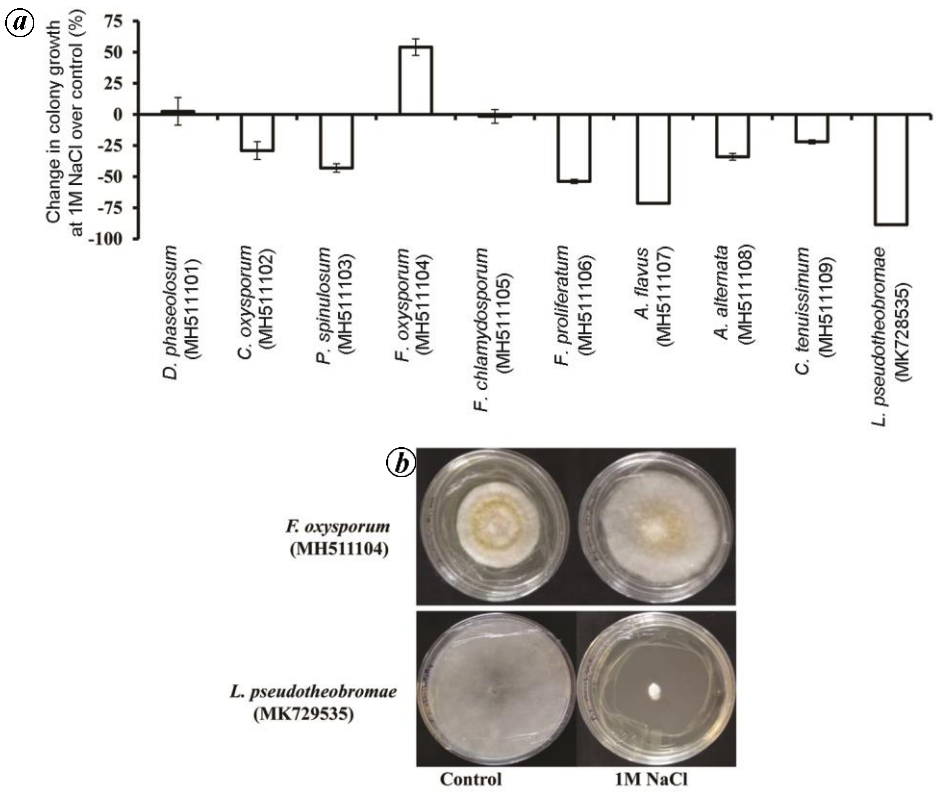

Figure 3. ***a***, Percentage change in growth of endophytic fungi colony at 1 M NaCl over control. The values are the means of $n = 3 \pm$ se. ***b***, Colony growth of salt tolerant, *F. oxysporum* (MH511104) and salt sensitive, *L. pseudotheobromae* (MK729535) on PDA plates under control and 1 M NaCl stress.

its ability to tolerate 2 M NaCl (approximately 11.7%). Most endophytes reported to-date from halophytes have tolerance levels ≤ 4% NaCl which is less than or equal to sea water salt concentrations[6,28]. A few though, have also reported endophytes with tolerance above 4% NaCl to as high as 26% NaCl (refs 26, 31). Endophytes associated with plants adapted to extreme habitats, such as hot deserts, salt marshes, arid habitats also exhibit tolerance to such environmental adversities[26,27,32]. In fact, because of their shorter generation time, it is suggested that

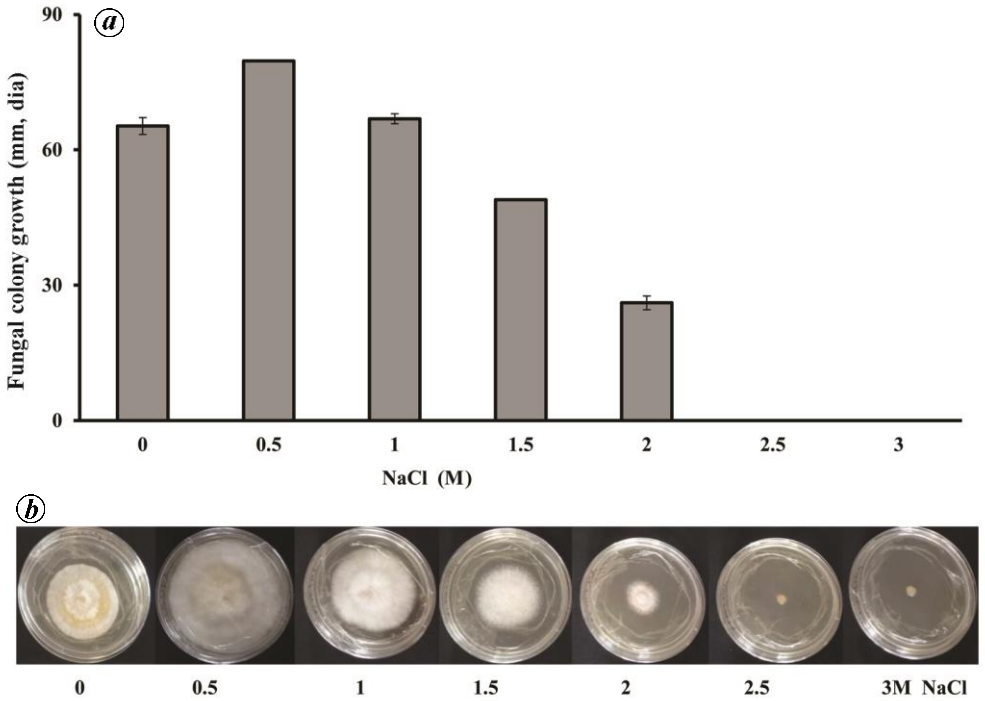

Figure 4. ***a***, ***b***, Growth (mm, dia) of fungal endophyte, *F. oxysporum* (MH511104) on PDA amended with different concentrations of NaCl (0, 0.5, 1, 1.5, 2, 2.5 and 3 M) after 5 days of inoculation. The values represented are means of $n = 3 \pm$ se.

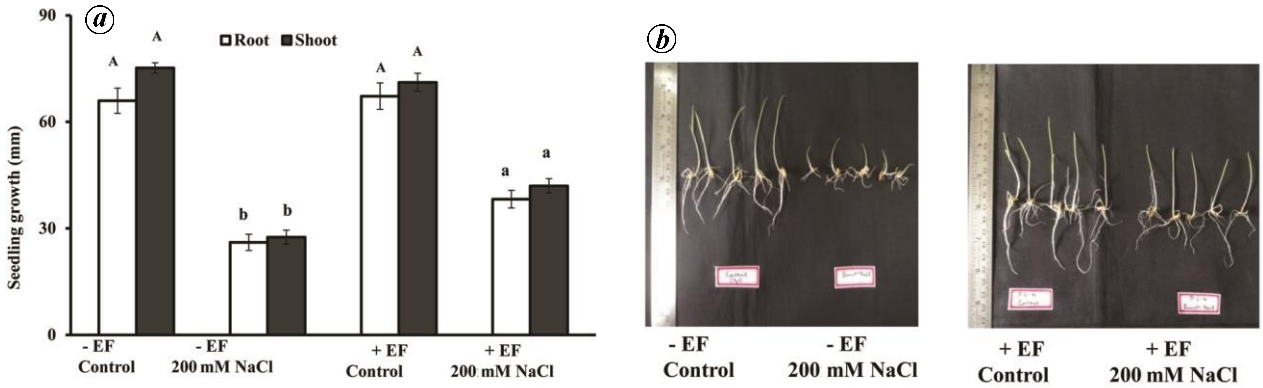

Figure 5. ***a***, Root and shoot length (mm ± se) of –EF and +EF paddy seedlings under control and 200 mM NaCl stress (data is the average of $n = 60$ seedlings). Letters above error bars indicate the significance level at $P \leq 0.05$. Capital letters and small letters indicate statistical significance between control and stress treatments respectively. ***b***, Phenotype of –EF and +EF paddy seedlings after five days of fungal inoculation under control and 200 mM NaCl.

endophytes might acquire adaptation to the stresses more rapidly than their respective host plants and furthermore even impart such tolerance to their hosts[29,32]. Rodriguez *et al.*[6] call this adaptation as 'habitat-adapted symbiosis' and showed that endophytes isolated from salinity-adapted plants, not only are salt tolerant but also confer tolerance to salt-sensitive paddy plants. Recent studies indicate that such a stress tolerance could be transferred by the endophyte even to non-host plants[33]. Though the mechanisms by which endophytes may confer salt tolerance to their host plants is not clear, it is suggested that it may involve the synthesis of host stress responsive hormones[34], upregulation of host stress responsive genes[35] and also by actively maintaining a low $Na^+ : K^+$ ratio[35].

In summary, our study demonstrated the importance of prospecting endophytes from plants adapted to extreme habitats to use them to improve crop growth in stressed environments. It would be interesting to examine the underlying physiological and molecular basis of such

cross-adaptations to make the endophyte-based strategy more robust as an alternative to conventional crop improvement programmes.

1. Beena, K. R., Ananda, K. and Sridhar, K. R., Fungal endophytes of three sand dune plant species of west coast of India. *Sydowia*, 2000, **52**(1), 1–9.
2. De-Souza, M. M., Madeira, A., Berti, C., Krogh, R., Yunes, R. A. and Cechinel-Filho, V., Antinociceptive properties of the methanolic extract obtained from *Ipomoea pes-caprae* (L.) R. Br. *J. Ethnopharmacol.*, 2000, **69**(1), 85–90.
3. Venkateasan, A., Prabakaran, R. and Sujatha, V., Phytoextract-mediated synthesis of zinc oxide nanoparticles using aqueous leaves extract of *Ipomoea pes-caprae* (L). R. Br. revealing its biological properties and photocatalytic activity. *Nano Technol. Environ. Eng.*, 2017, **2**(1), 8; https://doi.org/10.1007/s41204-017-0018-7
4. Manigauha, A., Kharya, M. D. and Ganesh, N., *In vivo* antitumor potential of *Ipomoea pes-caprae* on melanoma cancer. *Pharmacogn. Mag.*, 2015, **11**(42), 426.
5. Chan, E. W. C., Baba, S., Chan, H. T., Kainuma, M. and Tangah, J., Medicinal plants of sandy shores: A short review on *Vitex trifolia* L. and *Ipomoea pes-caprae* (L.) R. Br. *Indian J. Nat. Prod. Resour.*, 2016, **7**(2), 107–115.
6. Rodriguez, R. J. *et al.*, Stress tolerance in plants via habitat-adapted symbiosis. *ISME J.*, 2008, **2**(4), 404–416.
7. Shrivastava, P. and Kumar, R., Soil salinity: a serious environmental issue and plant growth promoting bacteria as one of the tools for its alleviation. *Saudi J. Biol. Sci.*, 2015, **22**(2), 123–131.
8. Munns, R. and Tester, M., Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.*, 2008, **59**, 651–681.
9. Thomson, M. J. *et al.*, Characterizing the Saltol quantitative trait locus for salinity tolerance in rice. *Rice*, 2010, **3**(2), 148; doi:org/10.1007/s12284-010-9053-8.
10. Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D. and Kursar, T. A., Are tropical fungal endophytes hyperdiverse?. *Ecol. Lett.*, 2000, **3**(4), 267–274.
11. Schulz, B., Wanke, U., Draeger, S. and Aust, H. J., Endophytes from herbaceous plants and shrubs: effectiveness of surface sterilization methods. *Mycol. Res.*, 1993, **97**(12), 1447–1450.
12. Booth, C., *The Genus Fusarium*, Commonwealth Mycological Institute, Kew Survey England, 1971, p. 237.
13. Domsch, K. H., Gams, W. and Anderson, T. H., *Compendium of Soil Fungi*, Academic Press (London) Ltd, 1980, vol. 1, p. 859.
14. Arx von, J. A., The genera of fungi sporulating in pure culture. Ganther, K. G. & J. Cramer, Vaduz, Liechtenstein, 3rd edn, 1981.
15. Huang, W. Y., Cai, Y. Z., Hyde, K. D., Corke, H. and Sun, M., Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. *Fungal Divers.*, 2008, **33**, 61–75.
16. Hammer, Ø., Harper, D. A. T. and Ryan, P. D., PAST: paleontological statistics software package for education and data analysis. *Palaeontol. Electron.*, 2001, **4**, 9.
17. Doyle, J. J. and Doyle, J. L., A rapid DNA isolation procedure for small quantities of fresh leaf tissues. *Phytochem. Bull.*, 1987, **19**, 11–15.
18. White, T. J., Bruns, T., Lee, S. J. W. T. and Taylor, J. L., Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications*, (eds Gelfand, M., Sninsky, D. and White, T. J.), Academic Press, New York, NY, USA,1990, vol. 18(1), pp. 315–322.
19. Higgins, K. L., Arnold, A. E., Miadlikowska, J., Sarvate, S. D. and Lutzoni, F., Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. *Mol. Phylogenet. Evol.*, 2007, **42**(2), 543–555.
20. Saitou, N. and Nei, M., The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, 1987, **4**(4), 406–425.
21. Kumar, S., Stecher, G. and Tamura, K., MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.*, 2016, **33**(7), 1870–1874.
22. Felsenstein, J., Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 1985, **39**(4), 783–791.
23. Zhang, Q., Zhang, J., Yang, L., Zhang, L., Jiang, D., Chen, W. and Li, G., Diversity and biocontrol potential of endophytic fungi in *Brassica napus*. *Biol. Control*, 2014, **72**, 98–108.
24. Suryanarayanan, T. S. and Kumaresan, V., Endophytic fungi of some halophytes from an estuarine mangrove forest. *Mycol. Res.*, 2000, **104**(12), 1465–1467.
25. Thiem, D., Piernik, A. and Hrynkiewicz, K., Ectomycorrhizal and endophytic fungi associated with *Alnus glutinosa* growing in a saline area of central Poland. *Symbiosis*, 2018, **75**(1), 17–28.
26. Manasa, K. M., Ravikanth, G., Nataraja, K. N. and Uma Shaanker, R., Isolation and characterization of endophytic fungi from saline habitat adapted plants. *Mysore J. Agric. Sci.*, 2015, **49**(2), 299–301.
27. Soares, M. A., Li, H. Y., Kowalski, K. P., Bergen, M., Torres, M. S. and White, J. F., Evaluation of the functional roles of fungal endophytes of *Phragmites australis* from high saline and low saline habitats. *Biol. Invasions*, 2016, **18**(9), 2689–2702.
28. Paranetharan, M. S., Thirunavukkarasu, N., Rajamani, T., Murali, T. S. and Suryanarayanan, T. S., Salt-tolerant chitin and chitosan modifying enzymes from *Talaromyces stipitatus*, a mangrove endophyte. *Mycosphere*, 2018, **9**(2), 215–226.
29. Yuan, Z. *et al.*, Specialized microbiome of a halophyte and its role in helping non-host plants to withstand salinity. *Sci. Rep.*, 2016, **6**, 32467; doi:10.1038/srep32467.
30. Indira, K. and Srinivasan, M., Diversity and ecological distribution of endophytic fungi associated with salt marsh plants. *Indian J. Geo. Mar. Sci.*, 2017, **46**(3), 612–623.
31. Liu, K. H. *et al.*, Morphological and transcriptomic analysis reveals the osmoadaptive response of endophytic fungus *Aspergillus montevidensis* ZYD4 to high salt stress. *Front Microbiol.*, 2017, **8**, 1789; doi.org/10.3389/fmicb.2017.01789.
32. Sangamesh, M. B. *et al.*, Thermotolerance of fungal endophytes isolated from plants adapted to the Thar Desert, India. *Symbiosis*, 2018, **75**(2), 135–147.
33. Rho, H., Hsieh, M., Kandel, S. L., Cantillo, J., Doty, S. L. and Kim, S. H., Do endophytes promote growth of host plants under stress? A meta-analysis on plant stress mitigation by endophytes. *Microb. Ecol.*, 2018, **75**(2), 407–418.
34. Asaf, S. *et al.*, Salt tolerance of *Glycine max*. L induced by endophytic fungus *Aspergillus flavus* CSH1, via regulating its endogenous hormones and antioxidative system. *Plant Physiol. Biochem.*, 2018, **128**, 13–23.
35. Abdelaziz, M. E., Kim, D., Ali, S., Fedoroff, N. V. and Al-Babili, S., The endophytic fungus *Piriformospora indica* enhances *Arabidopsis thaliana* growth and modulates Na^+/K^+ homeostasis under salt stress conditions. *Plant Sci.*, 2017, **263**, 107–115.

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