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1 **Fungal endophytes from salt adapted plants confer salt tolerance and promote growth**
2 **in Wheat (*Triticum aestivum* L.) at early seedling stage**

3 **Fungal endophytes mediated salt tolerance**

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17 **Abstract:**

18 With increasing human global population, increased yield under saline conditions is a
19 desirable trait for major food crops. Use of endophytes, isolated from halophytic hosts, seems
20 to be an exciting approach for conferring salt tolerance to a salt sensitive crop. Therefore, in
21 the current study, fungal endophytes were isolated from halophytic plants' roots and their
22 ability to withstand in vitro salt stress was evaluated. They could withstand upto 1M NaCl
23 concentrations and this tolerance was independent of their host or tissue source. When
24 inoculated on salt sensitive wheat seeds/seedlings several of the endophytes showed a
25 positive impact on germination and biomass related parameters upon salt stress, both *in vitro*
26 and under glasshouse conditions. One of the isolate from dicot plants (identified as
27 *Microsphaeropsis arundinis*) could successfully colonize wheat and promote its growth under
28 salt and no salt conditions. Amongst the fungal isolates that are known to be natural
29 endophytes of wheat, Chaetomium globosum was the best performing isolate which has been
30 reported as an effective biocontrol agent earlier. Based on the results of our preliminary
31 study, we suggest that these fungal endophytes could prove beneficial for salt stress tolerance
32 enhancement of wheat crop.

33 **Keywords:** endophytes, salt tolerance, halophytes, growth promoting activity,
34 *Microsphaeropsis arundinis,* wheat

35

36 Soil salinity is considered the scourge for plant growth and crop productivity
37 worldwide[1]. Approximately 1125 m ha of land throughout the world is affected by high
38 levels of salt due to intensive agriculture and desertification processes[2]. Increase in salinity
39 tolerance for the world's two major crops, wheat and rice, is an important goal as the world's
40 population is increasing more rapidly than the area of agricultural land[3]. Seed germination
41 and seedling growth of wheat, like other crops, has been found to be negatively affected by
42 salinity stress[4,5]. As a consequence, plant tolerance to salt, mainly to the sodium cation (Na^+),
43 is a desirable trait to be selected in cultivated crop plants. To overcome salinity stress,
44 tolerant variety can be developed through agronomical and breeding or advanced molecular
45 techniques, but these are time consuming and highly expensive. In this regard, one of the

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46 alternative approaches to achieve normal plant growth under salt stress is the efficient
47 utilization of endophytes[6].

48 Endophytes (endo = within, phyte = plant) represent an important component of the
49 plant microbiome and comprise of both bacteria and fungi. They are present in all plant
50 species asymptomatically but often promote host performance in terms of growth and
51 resistance to abiotic and biotic stresses. Endophytes isolated from plants growing in warm
52 soils and coastal saline soils indicate a high commercialization potential in agriculture by
53 providing increased crop yield in hot and salty water environments, respectively[7,8]. These
54 previous studies collectively show positive effects of endophytes on improving plant fitness
55 and survival under the different stress conditions, supporting the hypothesis that the effects of
56 endophytes on plant salt stress mitigation may be general among different plant taxa and
57 stress conditions. However, a well-structured study is needed to test this hypothesis.

58 To draw overall conclusions about the positives of endophytes for plant salt stress
59 tolerance, it is imperative to identify host-endophyte combinations that yield tolerance to salt.
60 In this regard, we isolated the endophytic fungi associated with halophytic plants growing in
61 coastal areas of Western Australia and evaluated their ability to tolerate NaCl stress. The
62 isolates which were tolerant to high concentrations of salt (1 M NaCl), were inoculated on
63 seeds of salt-sensitive wheat germplasm line to examine their ability to confer salt tolerance
64 to the new host. The results of the current study are important because they not only open up
65 exciting possibilities of using endophytes from salt adapted plants for mitigating salt stress in
66 agricultural crops but also in understanding the underlying biochemical and molecular basis
67 of plant-endophyte interaction.

68 MATERIALS AND METHODS

69 *Collection site and sampling*

70 Halophytic plants of eight species were collected from wild populations growing at
71 three coastal sites in Western Australia. Roots and rhizosphere soil of *Oxalis pes-caprae*
72 (soursop, *Oxalidaceae*), *Chenopodium album* (fat-hen, *Amaranthaceace*), *Elymus repens*
73 (couch grass, *Poaceace*), and an unidentified brassicaceous plant (*Brassicaceae*) were
74 collected at Collins Pool, Birchmont, located beside an estuary. Roots and rhizosphere soils
75 of *Salicornia quinqueflora* (beaded samphire, *Amaranthaceace*), *Juncus acutus* (rush,
76 *Juncaceae*), and an unidentified grass (*Poaceace*) were collected at Herron Point, Birchmont,
77 located beside the same estuary. Rhizosphere soil and stolons of *Ammophila arenaria*
78 (marram grass, *Poaceace*), and rhizome of *Posidonia australis* (sea grass, *Posidoniaceae*)
79 were collected from a beach located near the city of Bunbury, Australia (Table S1).

80 *Measurement of soil salinity and pH*

81 Soil salinity and pH were calculated in the field (Table S1). Five ‘5 cm diameter’
82 cores of soil were collected adjacent to sampled plants, with the exception of the seagrass
83 samples. Cores were taken to 10 cm depth and thoroughly mixed. A sample of 20 g of soil
84 was placed in a vessel and 100 mL of distilled water was added. The mixture was shaken
85 periodically over one hour, then allowed to stand for 30 min before measuring the salinity
86 (calculated from electrical conductivity) and pH using an EC8500 portable pH and
87 conductivity meter (Apera Instruments, Ohio) according to the manufacturer’s instructions. A
88 temperature compensation coefficient of 2%/^{o}C was used for calculating salinity. pH
89 measurements were later confirmed in the laboratory using an Orion Star A111 pH meter
90 (Thermo Fisher Scientific, Massachusetts).

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91 ***Isolation and culture of fungal endophytes***

92 Plant samples were rinsed under running tap water to remove surface debris and soil
93 particles. Fungal endophytes were isolated using a protocol described before[9]. Petri plates
94 were incubated at 25°C for 48 h and fungal colonies were counted. Colonization frequency
95 was estimated as follows:

$$\text{Colonization frequency (\%)} = \frac{\text{Total number of segments yielding fungus}}{\text{Total number of segments incubated}} \text{X}100$$

96 To obtain pure cultures of each fungal isolate, hyphal tips of colonies were transferred to Petri
97 plates containing 0.2x potato dextrose agar (PDA) supplemented with streptomycin sulfate
98 (0.1 mg mL^{-1}). Cultures were stored long-term at -80°C in 15% (v/v) glycerol.

99 ***Identification of fungal endophytes***

100 Morphological *viz*., colony color, mycelial texture and growth rate; and margin
101 characteristics were recorded for pure fungal cultures. If several isolates of similar
102 appearance were available from the same host plant then only two were chosen for molecular
103 identification. Genomic DNA was extracted[10] and Internal Transcribed Spacer (ITS) regions
104 were amplified by PCR using universal primers ITS1 and ITS4 or ITS4 and ITS5[11].
105 Amplified products were quantified and sequenced. Further, the obtained ITS sequences were
106 compared with those available on databases such as GenBank (NCBI) and UNITE[12] in order
107 to reveal their identity. Isolates were identified to the species level if their ITS sequences
108 shared ≥97% pairwise similarity with a named species from the databases analysed. When the
109 similarity percentage was 95-96%, only the genus name was accepted and for sequence
110 identities <95%, isolates were classified to the level of family (if available) or labelled as
111 'unidentified fungus' as described earlier[13]. Sequences were aligned using ClustalW and
112 percent similarity was obtained using the EMBL-EBI
113 (http://www.ebi.ac.uk/Tools/msa/mafft/) platform. Phylogeny was estimated using the
114 Maximum Likelihood (ML) method within MEGA v6.06 (http://www.megasoftware.net/)
115 after that 'Find Best DNA Models' was applied to determine the most appropriate model for
116 construction of respective ML phylogenies. Predicted tree branches were supported with
117 1000 bootstrap replications.

118 ***Evaluation of endophytic fungal isolates for salt tolerance***

119 Fungal isolates from each plant species were evaluated for tolerance to salt *in vitro*.
120 Endophytic fungal isolates were sub-cultured on PDA and allowed to grow for 7 d. A 5 mm^2
121 agar plug of mycelium was excised from the edge of the colony and used to inoculate potato
122 dextrose salt agar (PDSA) plates, which were PDA plates amended with 1.0 M NaCl. Fungal
123 colonies grown on PDA plates served as control. Cultures were incubated at 25°C in the dark.
124 Three replications were maintained for each treatment. The diameter of each mycelial colony
125 was recorded on the seventh day following plate inoculation. Diametrical growths of colonies
126 were measured at three different diameters per plate and the mean of these measures for
127 overall replications was calculated. Inhibition of growth under treatment on PDSA medium
128 was calculated as a percentage of growth of the same isolate growing on PDA medium.
129 Classification of salt tolerance of endophytic fungi was as described previously[14]. Highly
130 tolerant fungal endophytes were used for further studies.

131 ***In vitro screening of fungal endophytes for conferring salt tolerance to host***

132 The wheat genotypes obtained from Edwards's laboratory, SABC, Murdoch
133 University, Perth, Australia were initially screened for tolerance at different salt

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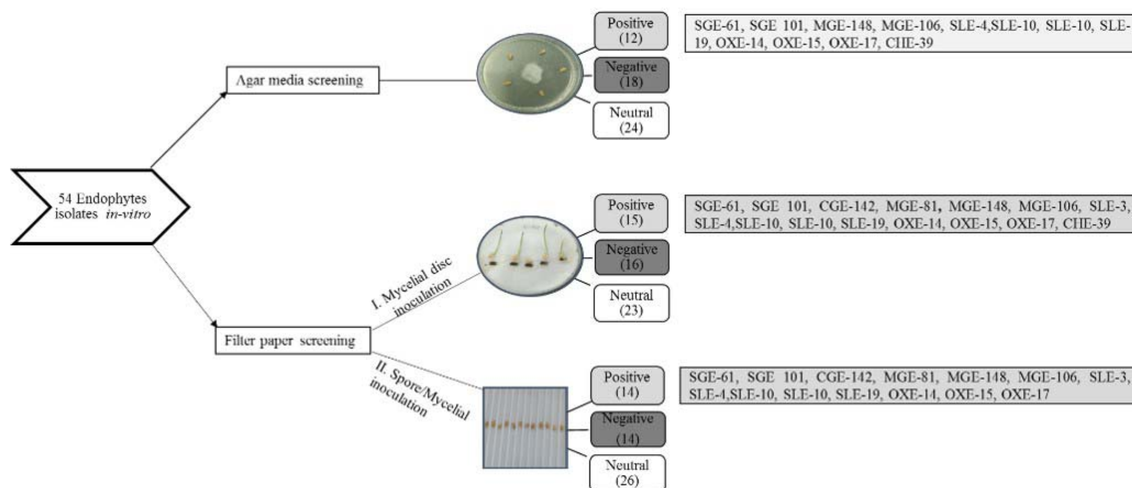
134 concentrations. All wheat genotypes were found highly sensitive to salt (NaCl) at 150 mM
135 concentration (data not shown), therefore this concentration was used in the current study.
136 Among the wheat genotypes GP#15, with agronomical superiority, was used for further
137 studies. The selected fungal endophytes were evaluated *in vitro* for their ability to impart
138 salinity tolerance to a salt-sensitive wheat genotype at 150 mM NaCl. Two different
139 methodologies were employed for the *in vitro* stress tolerance studies: agar media based
140 method and filter paper methods as illustrated in Figure 1.



141
142 **Fig. 1** Schematic representation of different approaches employed for *in vitro* screening of
143 fungal endophytes, isolated from halophytic species, that conferred salt (150 mM NaCl)
144 tolerance to wheat seedlings (observation were made at 10 days after treatment). The number
145 in the parenthesis indicates counts of the endophyte isolates showing respective interaction:
146 **Positive**= enhanced tolerance, **Negative** = decreased tolerance or retarded growth upon
147 inoculation, **Neutral** = no measurable influence. Names of the isolates have been provided in
148 the box

149 **i. Agar media based test**

150 Each fungal endophyte was applied individually to wheat (GP#15) seeds according to
151 method described[15]. Briefly, a 5 mm^2 agar plug, cut from the margins of the 10 day old
152 colony were placed, hyphal side down, in the middle of each Petri dish containing 2/4th
153 strength PDA media amended with 150mM NaCl (each Petri dish was filled 2/3rd with media
154 and assumed variation was minimized by replication) and then incubated at 25°C. Five
155 surface sterilized seeds were placed at a distance equivalent to 24 h and 72 h grown culture
156 plates for fast growing and slow growing isolates respectively. The Petri dishes were sealed
157 with parafilm and then incubated at room temperature (25°C). 10 days post-inoculation (dpi),
158 the parafilm was removed and observations on host endophyte reaction were recorded. The
159 control treatments contained no fungus. Each fungal inoculation or control was replicated at
160 least three times.

161 **ii. Filter paper based test**

162 Long strips were made from filter paper (Whatman 10312209, Grade 598) and two
163 strips per furrow were placed in plastic plate with 12 furrows as shown in Figure 1. Sodium
164 chloride solution (250 µl of 150 mM NaCl) was added in each furrow and surface sterilized
165 wheat seed were placed in center of each furrow. The plates were sealed with parafilm
166 (Parafilm® M, P7793, Sigma) and incubated at 25°C in an inclined position to facilitate
167 downward root movement. Once radicle had grown 5 mm in length, a 3 mm^2 agar plug made

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168 from the growing edge of endophytic fungal colony was placed along the radicle of each
169 seedling and plates were sealed again with parafilm and incubated as before. At 10 dpi, the
170 parafilm was removed and host-endophyte reaction was recorded. The control treatments
171 with an agar plug contained no fungus.
172 Using agar media based method, germination kinetics parameters such as germination
173 percentage (G%) and mean germination time (MGT) and biomass related parameters such as
174 root length, shoot length and seedling fresh weight were recorded for hosts inoculated with
175 endophytes (n=13), referred to as endophyte inoculated (EI) seeds hereafter. Control plates
176 contained non-inoculated (NI) wheat seeds placed on media with or without salt.

177 ***Root colonization by fungal endophytes***

178 Trypan blue (0.01% w/v) staining was used to identify fungal mycelium within root
179 tissues using a method used earlier[16] with suitable modifications applicable to root tissues.
180 Briefly, seedlings inoculated on filter paper (Method I and II) were collected 15 dpi. Roots
181 were cut into approximately 0.5 cm segments and were cleared with acetic acid:ethanol (1:3
182 v/v) solution for 12 h. A second tissue clearing was done by soaking tissues in acetic
183 acid:ethanol:glycerol (1:5:1 v/v/v) solution for 5 h. The samples were subsequently incubated
184 overnight in a staining solution of trypan blue. Stained tissues were rinsed with 60% sterile
185 glycerol and stored in it until examination. Specimens were examined under an Olympus BX
186 51 optical microscope (Olympus, Japan). Five to ten segments were assessed per endophyte
187 inoculation treatment.

188 ***Glasshouse based evaluation of fungal endophytes for conferring salt tolerance to host***

189 Based on the ability of isolates in conferring salt tolerance to wheat *in vitro*, 11
190 isolates were further selected (SLE-6, SLE-10, SLE-19, OXE-14, OXE-17, SGE-60, SGE-61,
191 MGE-81, MGE-106, MGE-148 and CGE-142) for glasshouse experiment. Spore suspensions
192 were prepared from 10 day old cultures of highly salt tolerant fungal isolates growing in 2/4th
193 strength potato dextrose broth and incubated on a shaker. The mycelial pellicle was washed
194 in sterile water to remove residual broth, then macerated in a blender and filtered through
195 sterile cotton wool. The number of spores was counted using a haemocytometer and diluted
196 to $1x10^7$ spores mL^{-1}. Wheat seeds were soaked in spore suspension, of individual endophyte,
197 overnight after which they were taken out from the suspension and shade dried. Five seeds
198 were sown in perforated pots filled with perlite and sand (3:2). The pots were placed in
199 plastic trays either containing 150 mM NaCl solution or water. Each tray contained 6 pots
200 and 500 ml salt solution or water (each pot served as one replication). Similarly seeds soaked
201 in water were sown in six separate pots and placed in a tray containing water and served as
202 control. Once in 3 days salt solution was replaced with fresh salt solution (to avoid salt
203 accumulation in trays they were washed thoroughly and solution was replaced). Results were
204 reconfirmed by repetition of experiment.

205 ***Evaluation of physiological and biomass related parameters of host***

206 **i. Chlorophyll content (CC)**

207 Chlorophyll content was measured from fully expanded leaves (1st leaf as shown in
208 Figure S1) of seedlings by a hand-held chlorophyll meter (CCM-200 plus, Opti-Sciences Inc.,
209 Hudson, NH, USA). Three seedlings per pot were investigated. A total of eighteen seedlings
210 were considered from each treatment and averaged value was taken as CC per seedling.
211 Chlorophyll data measurement was carried out 7, 11 and 15 days after stress was imposed,
212 just before the plants were harvested.

213 **ii. Relative Water Content (RWC) and Biomass**

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214 Fully expanded leaf of wheat seedling was used to estimate RWC. A total of 10 leaves
215 were harvested randomly from six pots in each treatment. The leaves were placed in
216 polythene bags and transported to the laboratory as quickly as possible in order to minimize
217 water losses due to evaporation and were also weighed immediately to obtain fresh weight
218 (Fw). Then, leaves were soaked in distilled water in test tubes for 24 h at 4 °C in the dark,
219 and turgid weight (Tw) was noted. Subsequently, samples were dried in the oven at 70 °C for
220 24 h, and dry weight (Dw) was measured. RWC of seedling was determined as:

221 $$\mathbf{RWC = (Fw - Dw)/(Tw - Dw) \times 100}.$$

222 A total of six seedlings were selected per treatment for the estimation of seedling
223 biomass parameters viz., root length, shoot length and root and shoot dry weight. Seedlings
224 were divided into roots and shoots, and soil was washed from roots by hand. Samples were
225 desiccated for 48 h at 80 °C, and dry weight (mg) was recorded. Also, root to shoot ratio was
226 calculated based on their length.

227 ***Statistical analysis***

228 To describe the variability, several simple univariate analysis including means, ranges
229 and variance were calculated. Coefficients of variation (CV%) was also calculated from the
230 variance components and the overall means for all the investigated treatments. Clustering of
231 different treatments based on the CC was carried out using 'Fastcluster' package of R
232 statistical software (version 3.4.4) with squared Euclidean distance as a measure of
233 dissimilarity and incremental sums of squares as a grouping strategy[17]. Data of all characters
234 were standardized to a mean of zero and variance of one and Principal Component Analysis
235 (PCA) was performed. First, second and third principal component axes scores were plotted
236 together to visualize the effect of different treatments simultaneously.

237 **RESULTS**

238 All halophytic plants examined were found to be colonized by multiple culturable
239 fungal endophytes. Two hundred and forty two fungal isolates were obtained from 320 plant
240 specimen and their colonization frequency ranged from 63% to 96% (Figure S2). A high
241 number (96%) of endophytic fungi were isolated from the root tissues of *J. acutus* and *S.*
242 *quinqueflora* plants (Figure S2). Pure fungal cultures were initially grouped according to their
243 morphological (*viz*., colony color, mycelial texture and growth rate) and margin
244 characteristics.

245 ***Endophytes showed differential response to salinity in vitro***

246 One hundred and thirty fungal isolates were screened for their responses to 1.0 M
247 NaCl *in vitro*. Based on the degree of inhibition of radial growth on PDSA medium compared
248 to PDA medium, fungal isolates were grouped as highly-tolerant, tolerant, moderately-
249 tolerant, or sensitive (Table S2) as described earlier[14]. Most isolates (58) were grouped into
250 the moderately-tolerant category, followed by 39 isolates that were tolerant and 27 that were
251 highly-tolerant. The growth of 6 isolates was severely inhibited on PDSA therefore they were
252 categorised as sensitive. Endophytes originating from monocotyledonous and dicotyledonous
253 halophytes differed in their salt tolerance as shown in the Figure 2. Most isolates from
254 dicotyledonous halophytic hosts had moderate to high salt tolerance whereas most
255 endophytes from monocotyledonous hosts had moderate tolerance. Among the halophytes,
256 sea grass, a species constantly immersed in seawater (~550 mM NaCl), was colonised with
257 the most highly salt-tolerant endophytes. Moreover, isolates inhabiting the same host also
258 showed differential levels of salt tolerance.

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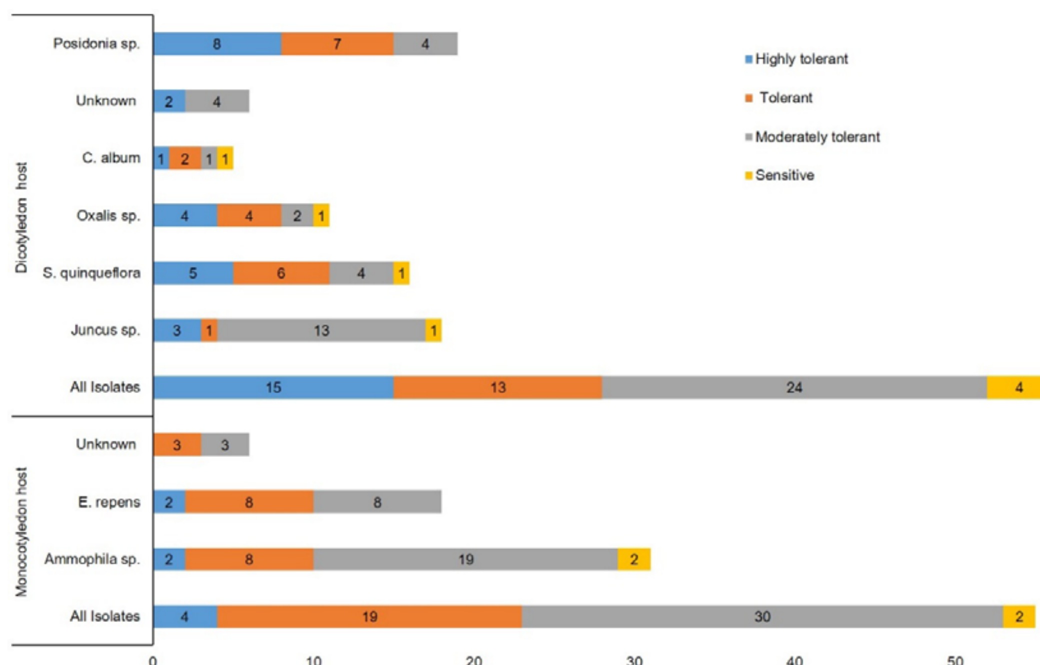

259
260 **Fig. 2** Categorization of endophytes originating from monocotyledonous and dicotyledonous
261 halophytes based on the difference in their salt tolerance

262 ***Fungal endophytes inhabiting halophytes belonged to highly diverse genera***

263 Based on morphological characteristics and inherent salt tolerance, fifty-four
264 representative fungal isolates were selected for molecular identification, where several
265 isolates of similar appearance were isolated from the same host plant, only two isolates were
266 chosen for molecular identification. The chosen isolates were identified based on ITS-
267 amplicon sequencing results followed by database similarity search. The ITS sequences
268 obtained have been deposited in the NCBI GenBank (Accession No. MK431041-MK431094;
269 Table S3). Database similarity search revealed that diverse fungal flora had colonized the
270 halophytic hosts used in the study. Twenty isolates could be identified completely i.e. upto
271 species level with some unidentified to the genus level (Table S3). All endophytes isolated
272 from halophytes were members of phylum Ascomycota and most of them belonged to
273 subphylum *Pezizomycotina* (48), and were distributed in three classes viz., *Dothideomycetes*
274 (17) *Eurotiomycetes* (7) and *Sordariomycetes* (24). Among the fungal orders, *Hypocreales*
275 (15), *Pleosporales* (17) and *Eurotiales* (7) were the most highly represented (Figure 3).
276 Dominant genera identified in this study were *Alternaria*, *Chaetoium*, *Fusarium* and
277 *Penicillium*, whereas genera that were represented by only one or a few isolates were
278 *Aquanectria*, *Aspergillus*, *Bipolaris*, *Clonostachys*, *Didymella*, *Didymosphaeria*, *Microascus*,
279 *Paraconiothyrium*, *Paraphaeosphaeria*, *Phaeosphaeria*, *Phoma*, *Phomopsis*,
280 *Plectosphaerella*, *Setosphaeria*, *Soradria* and *Trichoderma*. The six isolates that could not be
281 identified were classified as *Incertae sedis*. Further, phylogeny revealed that highly salt-
282 tolerant endophytic isolates grouped into a single cluster (Figure 4) indicating that they may
283 share some similarity at genetic level. No phylogenetic pattern was however evident with
284 regard to plant tissue type or host species (data not shown).

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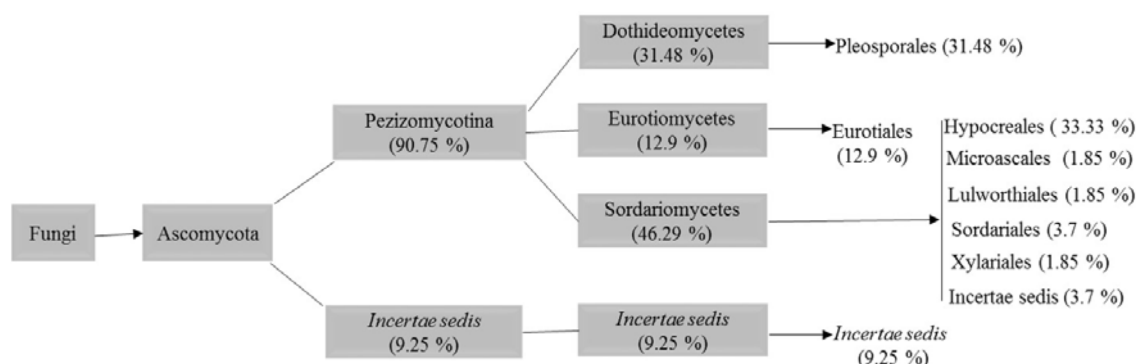

Fig. 3 Schematic representation of phylogenetic placement of 54 fungal species identified from ITS sequences of endophytes isolated from different halophytic species. Classification follows Hibbett et al. (2007)

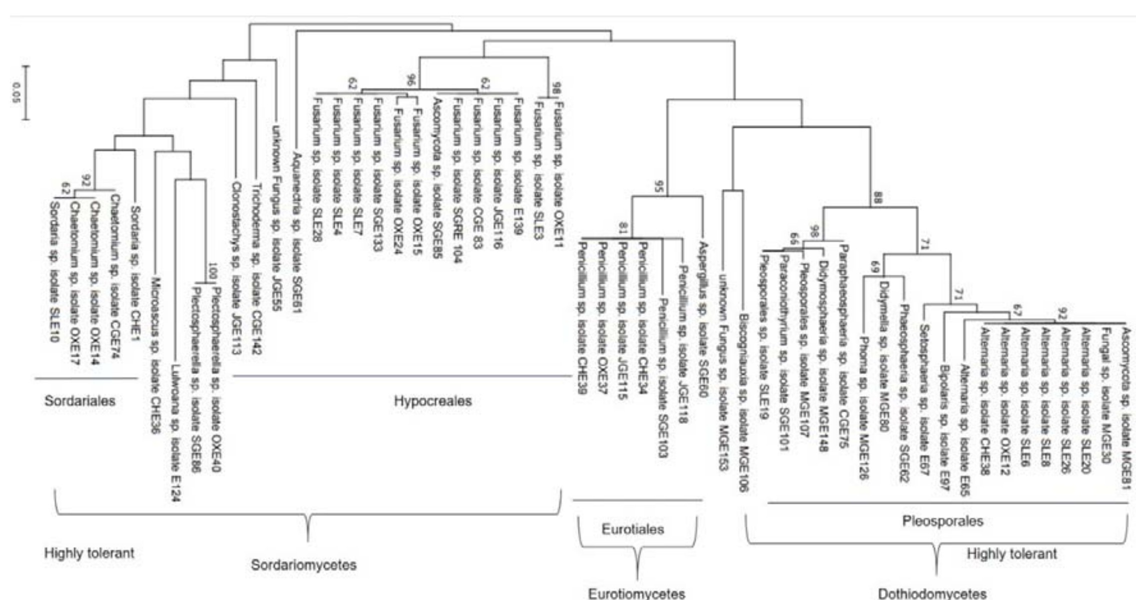

Fig. 4 Phylogenetic analysis of fungal endophytes isolated from halophytic hosts revealed that highly salt-tolerant endophytic isolates grouped into a single cluster

Fungal endophytes isolated from different tissues showed positive impact on salinity tolerance of wheat in-vitro

Seeds of the salt-sensitive wheat genotype (GP#15) were inoculated with 54 endophytes and the performance was evaluated on 150 mM NaCl using different approaches (Figure 1). Isolates enhancing seedling salt stress tolerance had been isolated from all type of tissues used under study. However, on agar method 21 % of isolates from the roots had positive impact whereas on filter paper method, 27 % of isolates from stolons had more positive impact (Figure S3). Based on their positive impact on seedling performance under salinity, some of these isolates were used for further analysis. Roots of EI seedlings were examined under a light microscope for the proof of endophytic colonization. Stained roots highlighted the presence of a network of hyphae, most of which penetrated the intercellular spaces of the root (Figure 5). The pure culture of some of these isolates are shown in Figure S4.

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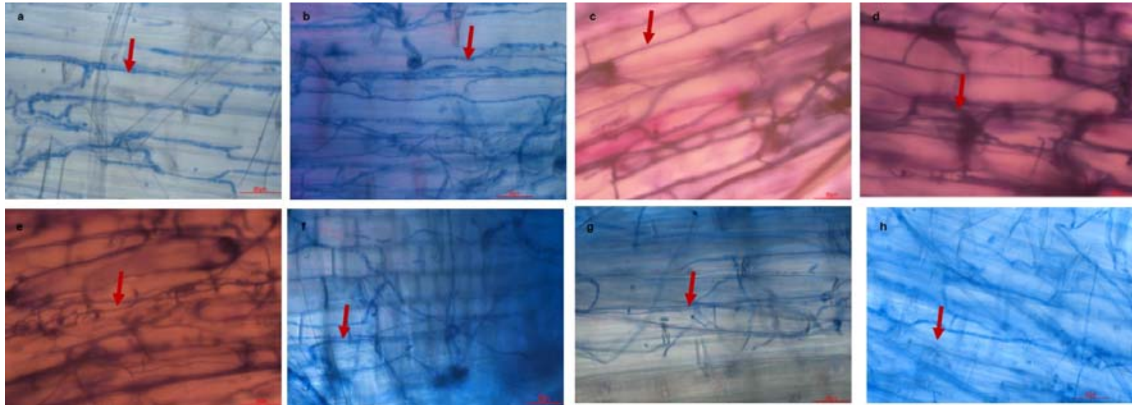
305 

306 **Fig. 5** Colonization by the fungal isolates inside the root tissue of salt sensitive wheat
307 inoculated in the filter paper screening test. The red-coloured arrow head indicates the
308 presence of fungal mycelia as observed under a compound microscope stained after trypan
309 blue staining a) *Trichoderma atroviride* b) *Alternaria infectoria* c) *Alternaria chlamydospora*
310 d) *Microsphaeropsis arundinis* e) *Didymosphaeria variabile* f) *Chaetomium globosum* g)
311 *Chaetomium globosum* h) *Chaetomium globosum*

312 ***Fungal endophytes promotes growth of wheat at early seedling stage under saline***
313 ***conditions***

314 Seeds of the salt-sensitive wheat genotype (GP#15) were treated with promising
315 endophytes (n=13) and the performance of the seedlings was analyzed both *in vitro* and under
316 glasshouse conditions after subjecting them to salt stress (150 mM NaCl).

317 Observations of *in vitro* assay revealed that G% of EI seeds placed on salt containing
318 media (SCM) ranged from 88.8 to 97.7%. The NI seeds showed 91% germination on SCM
319 and 97.7% on media without salt (MWS) indicating a higher germination rate in EI seeds
320 than NI seeds on SCM. The EI seeds placed on SCM showed mean germination time (MGT)
321 lesser than NI seeds on the same media, indicating that EI seeds germinated faster than NI
322 seeds when placed on SCM. However, the least MGT was recorded for NI seeds placed on
323 MWS (Figure 6).

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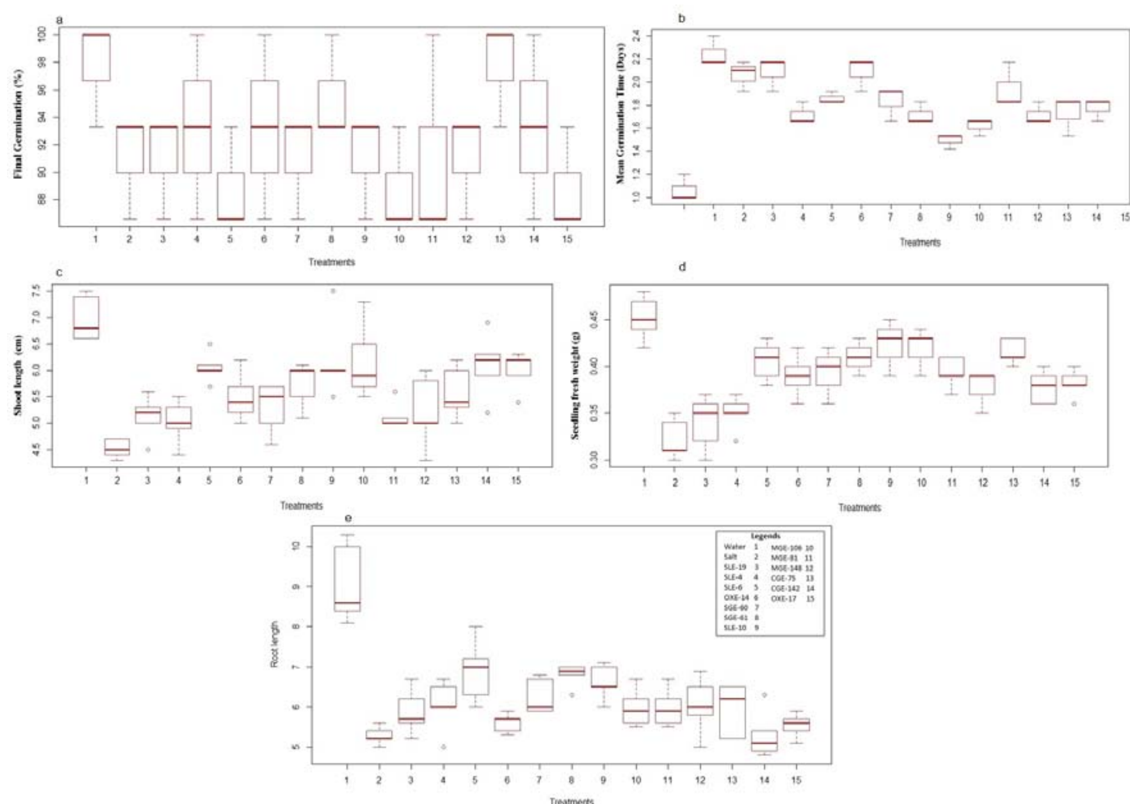

Fig. 6 Measurement of germination kinetics parameters and seedling biomass related parameters after subjecting the endophyte inoculated wheat (GP#15) seeds to salt stress (NaCl 150 mM) *in vitro*

Similar trends were observed for seedling biomass related parameters such as root length, shoot length and seedling fresh weight. The EI seedlings showed higher biomass as compared to NI seedlings on SCM (Figure 6). All the endophytic isolates improved the performance of the wheat seedlings under salt stress, however, we selected 11 best performing isolates for the glass house based studies (Table 1).

Table 1 Endophytic fungal isolates identified and used in the current study for evaluating their ability to confer salt (150mM NaCl) tolerance to wheat seeds/seedlings *in vitro* or in glass house respectively.

S. No.	Isolate code	Identity based on ITS sequencing and database similarity search	Host species	Salt tolerance	Accession no.
1.	SLE-6	*Alternaria chlamydospora*	*Salicornia quinqueflora*	Highly tolerant	MK431069
1.	SLE-19	*Microsphaeropsis arundinis*		Highly tolerant	MK431073
2.	SLE-10	*Chaetomium globosum*		Highly tolerant	MK431072

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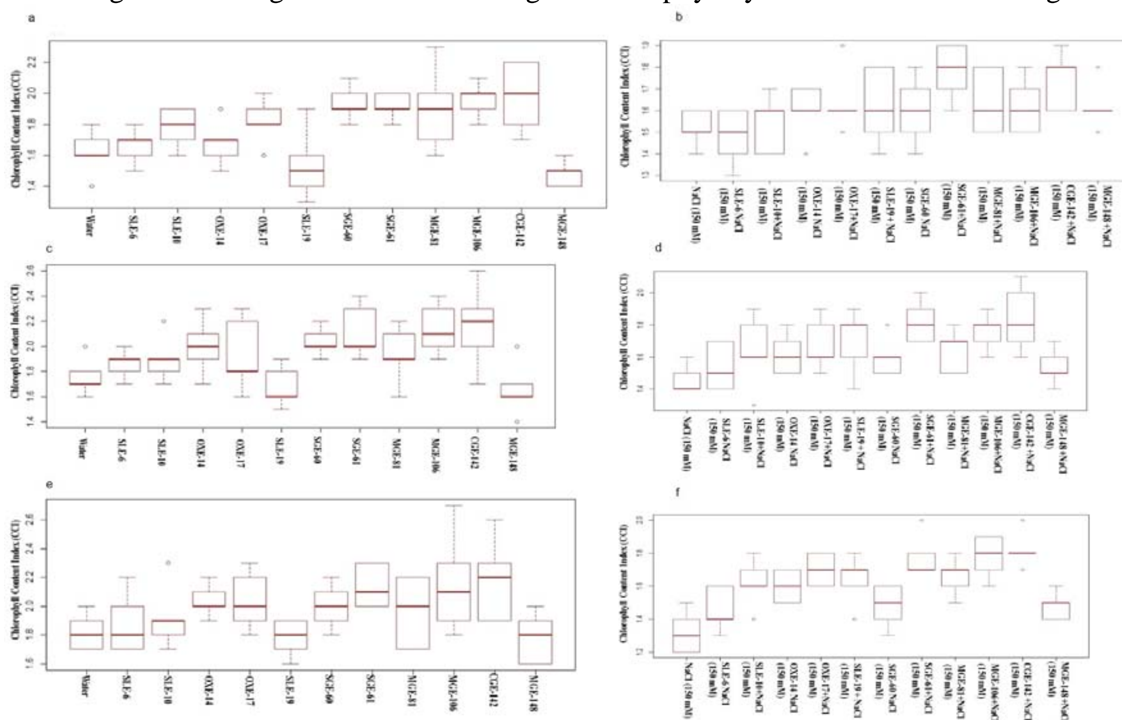
3.	OXE-14	*Chaetomium globosum*	*Oxalis pes-caprae*	Tolerant	MK431079
4.	OXE-17	*Chaetomium globosum*			MK431081
5.	SGE-60	*Aspergillus ochraceus*	*Posidonia australis*	Highly tolerant	MK431046
6.	SGE-61	*Aquanectria penicillioides*		Highly tolerant	MK431047
7.	MGE-81	*Alternaria infectoria*	*Ammophila arenaria*	Highly tolerant	MK431061
8.	MGE-106	Unknown fungal sp.		Highly tolerant	MK431062
9.	MGE-148	*Didymosphaeria variabile*		Tolerant	MK431065
10.	CGE-142	*Trichoderma atroviride*	*Elymus repens*	Tolerant	MK431058

For EI seedlings grown under glasshouse conditions, CC was measured at 7, 11 and 15 days after stress (das) treatment (Figure 7) to study the effect of duration of stress on CC of leaves. Chlorophyll estimation was also performed for NI seedlings grown in solution with or without salt at the same time points. Higher CC was observed in seeds treated with fungal isolate CGE-142, especially as the duration of stress increased (11 and 15 das). Similarly, CGE-142 inoculation enhanced the CC of seeds grown in solution without salt (SWS). These values were higher than the CC of NI seeds grown in SWS or salt containing solution (SCS), indicating that the fungal isolate induced higher chlorophyll synthesis in wheat seedlings.



347 **Fig. 7** Measurement of chlorophyll content index of endophyte inoculated wheat (GP#15) seedlings at 7 (a, b), 11 (c, d) and 15 (e, f) days of salt
348 stress (b, d, f) or no stress (a, c, e) under glass house condition

349 All the fungal endophytic isolates improved the RWC of wheat seedlings as compared to NI seedlings when grown in SCS (Table 2). The
350 highest RWC was observed in seedlings inoculated with CGE-142. With regard to biomass-related parameters, seedlings inoculated with SLE-
351 10 exhibited root and shoot biomass (fresh weight, dry weight and length) as well as root to shoot ratio higher than NI seedlings grown in SCS.
352 However, similar trend was observed in seedlings inoculated with CGE-142 in the absence of salt (Table 3).

353 **Table 2** Measurement of biomass related parameters of endophyte inoculated wheat (GP#15) seedlings grown under salt stress condition in the
354 glass house

Isolate code	**RWC (%)**	**% change over control**	**RDW (g)**	**% change over control**	**SDW (g)**	**% change over control**	**RL (cm)**	**% change over control**	**SL (cm)**	**% change over control**	**RSR**	**% change over control**
SLE-6+NaCl	95.0ab	5.62	0.07d	-37.15	0.08h	-32.88	8.76def	6.07	12.4f	-7.83	0.74ab	20.04
SLE-10+NaCl	93.78b	4.28	0.11a	0.76	0.13cd	9.96	9.70bcdef	18.69	14.8de	9.87	0.66abcd	10.46
OXE-14+NaCl	95.0ab	5.61	0.07d	-37.43	0.11ef	-5.26	9.20cdef	11.63	16.4cd	21.20	0.56def	-8.17
OXE-17+NaCl	90.94c	1.15	0.08cd	-28.55	0.12de	0.38	12.20a	46.44	17.6bc	30.86	0.71abc	15.27
SLE-19+NaCl	90.90c	1.10	0.09bc	-19.07	0.14bc	20.27	10.30bcd	23.95	18.2bc	34.56	0.57cde	-6.31
SGE-60+NaCl	95.0ab	5.63	0.08cd	-27.76	0.09gh	-23.24	8.90cdef	7.94	14.8ed	8.41	0.62bcd	0.23
SGE-61+NaCl	90.9c	1.10	0.10ab	-10.84	0.10fg	-15.79	10.56abc	27.48	17.3bc	28.43	0.62bcd	1.58
MGE-81+NaCl	95.82ab	6.57	0.09bc	-18.61	0.10fg	-13.79	11.10ab	32.61	14.7def	9.03	0.76a	24.96
MGE-106+NaCl	96.32a	7.11	0.04e	-63.45	0.15ab	28.96	8.06f	-3.77	18.9ab	39.50	0.43f	-31.18
CGE-142+NaCl	96.02a	6.78	0.04e	-63.72	0.16a	35.65	9.80bcde	19.05	20.9a	54.10	0.47ef	-22.87
MGE-148+NaCl	91.28c	1.48	0.07d	-37.43	0.10fg	-15.79	8.20ef	0.10	13.0ef	-3.97	0.63abcd	3.61
NaCl (150 mM)	90.0c		0.11a		0.12de		8.36ef		13.6ef		0.62bcd	

355 RWC: Relative water content, RDW: Root dry weight, SDW: Shoot dry weight, RL: Root length, SL: Shoot length, RSR: root to shoot ratio based on length.
356 Same letters indicate statistically insignificant differences ($p>0.05$)

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358 **Table 3** Measurement of biomass related parameters of endophyte inoculated wheat (GP#15) seedlings grown under no stress condition in the
359 glass house

Isolate code	**RWC (%)**	**% change over control**	**RDW (g)**	**% change over control**	**SDW (g)**	**% change over control**	**RL (cm)**	**% change over control**	**SL (cm)**	**% change over control**	**RSR**	**% change over control**
SLE-6	88.29b	11.31	0.12bc	7.09	0.13de	-12.58	12.6e	**-9.98**	14.8de	-6.29	0.85cde	-3.48
SLE-10	88.39b	11.44	0.12bc	11.09	0.14cd	-6.24	14.9dc	6.21	15.0de	-4.92	1.01bc	13.78
OXE-14	88.57b	11.67	0.14a	**29.54**	0.13e	-13.89	16.3bc	16.61	14.3de	-9.28	1.18a	**32.37**
OXE-17	88.37b	11.42	0.13ab	17.43	0.16ab	5.52	13.58de	-2.78	18.2bc	15.30	0.75de	-15.36
SLE-19	91.89a	15.88	0.12bc	7.09	0.12e	**-21.20**	17.2ab	22.82	16.0cd	1.18	1.07ab	20.91
SGE-60	92.31a	**16.42**	0.13ab	17.43	0.13de	-12.83	14.9dc	6.00	16.8bcd	6.14	0.91c	3.04
SGE-61	88.37b	11.43	0.14a	27.49	0.14cd	-6.24	17.3ab	23.64	18.8b	19.13	0.94bc	5.58
MGE-81	84.61c	6.69	0.14a	27.09	0.16ab	5.52	17.4ab	23.76	18.06bc	14.32	0.96bc	8.26
MGE-106	88.56b	11.67	0.12bc	6.43	0.17a	**13.37**	13.4de	-4.15	18.8b	19.09	0.71e	**-19.30**
CGE-142	91.85a	15.83	0.13ab	16.98	0.16ab	5.52	18.6a	**32.80**	21.9a	**38.91**	0.85cde	-3.97
MGE-148	91.67a	15.59	0.11c	0.22	0.12e	**-21.20**	12.64e	-9.73	13.0e	**-17.72**	0.99bc	10.76
Mock	79.31d		0.11c		0.15bc		14.04de		15.8cd		0.89cd	

360 RWC: Relative water content, RDW: Root dry weight, SDW: Shoot dry weight, RL: Root length, SL: Shoot length, RSR: root to shoot ratio based on length.
361 Same letters indicate statistically insignificant differences (p>0.05)

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363 **DISCUSSION**

364 The ability to tolerate briny water is essential for wild plants that live in coastal and
365 marine environments. Such halophytic plants appear to associate widely with fungi as evident
366 from the wide range of fungi described from marine-influenced systems of coastal sand
367 dunes, mangroves, seagrass and estuaries. Although, the roles played by these fungal
368 endophytes in salt tolerance of halophytes could be dependent on several factors[18] it can be
369 expected that their association with such hosts would provide them salt tolerance too[19].
370 Therefore, in the current study, we isolated fungal endophytes from halophytic plants
371 and tested their response to a high-salt environment *in vitro*. We challenged the endophytes
372 with a NaCl concentration (upto 1M NaCl) almost twice that of seawater. This concentration
373 reduced the growth rate of all isolates, but for many, growth inhibition was <50% that of low
374 salt conditions, an indication of tolerance to high osmotic gradients. We are aware that the
375 environment on PDSA medium is not likely to be equivalent to conditions in the interstitial
376 spaces between cells of salt-tolerant plants due to two reasons. Firstly, on a solid medium
377 mycelium is not immersed in an aqueous environment. Secondly, plants actively pump Na^+
378 and Cl^- ions from the roots, they compartmentalize salts in vacuoles, and they develop
379 osmotic tolerance (involving long-distance signalling) to cope with saline environments[20].
380 Thus, the salinity experienced by the fungus within the plant may be less than the external
381 salt concentration. Hence, if the fungal isolates could tolerate high salt concentration *in vitro*,
382 they can be expected to tolerate salt stress *in planta* also. As reported earlier, the levels of
383 tolerance to salt by endophytes mainly depends on genetic factors of the fungus species and
384 host habitat[21], the range of external salinity and accumulation of osmo-protectants in the
385 cytoplasm[18,22,23]. Therefore, the observed variations in salt tolerance of fungal endophytes
386 isolated from different hosts as well as the same host could be due to their genetic
387 constituents or their interaction with halophytic hosts. This finding is in line with earlier
388 studies that showed halophyte microbiomes exhibit differential levels of salt tolerance[24,25,26].
389 Moreover, all the highly salt tolerant isolates grouped together in a single cluster upon
390 phylogenetic analysis indicating underlying similarities between them.
391 It is unclear if salt tolerance of fungal endophytes isolated in the current study
392 corresponded to their possible roles in mediating salinity tolerance in their naturally salt-
393 adapted host plants, but it would have been interesting to observe their effect on growth of
394 salt-sensitive crop plants. Therefore, we selected 54 isolates, based on their inherent salt
395 tolerance, and inoculated them on salt sensitive wheat (GP#15) seeds. Mostly they exerted a
396 highly positive impact on GP#15 as evaluated by various *in vitro* techniques. These isolates
397 were then identified based on their ITS region sequences and found to belong to diverse
398 genera (Figure 3, Table S3). Earlier, endophytes isolated from mangrove leaves have been
399 reported to belong to *Acremonium, Phomopsis, Phyllosticta*, and *Sporormiella*[27], *Diaporthe*[28],
400 *Bruguiera*[29], *Aspergillus* species and others[30]. Only a few of these genera were represented in
401 our study, the difference could have been due to different tissue from where endophytes have
402 been isolated, for example we isolated endophytes from roots, however the earlier report had
403 used leaves of halophytic plants.
404 Further, some of these isolates (n=11) were selected for evaluating their growth
405 promoting activity on GP#15 when subjected to salt stress *in vitro* and under glasshouse
406 conditions. Their effect on germination kinetics of GP#15 seeds *in vitro* was also recorded.
407 Germination related parameters such as G% and MGT were better for EI wheat seeds than NI
408 seeds placed on SCM. Our results also showed that the endophytes inhabiting wheat plants
409 not only promote the growth of the seedling but also confer salt tolerance to the host as
410 compared to the NI control. This was found to be associated with an altered CC and RWC of
411 the wheat seedlings as well as enhanced biomass. Notably, the endophyte treatment provided

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412 an advantage to the wheat seeds with regard to their germination and biomass related
413 parameters when exposed to saline conditions. Endophytic association is a promising
414 approach to enhance salt tolerance, although specific mechanisms for this are unclear. The
415 presence of endophytes may stimulate inherent plant responses to salinity and/or provide
416 fungus derived compounds that mediate the stress response. In barley, the presence of the
417 endophyte *Piriformospora indica* induced elevation of ascorbic acid and antioxidant enzymes
418 in roots under salt stress[31]. *Phoma glomerata* and *Penicillium* sp. endophytes in dwarf rice
419 secreted gibberellic acid and indole acetic acid to promote growth under saline conditions[32].
420 The basis for selecting these 11 isolates was mainly driven by their salt tolerance
421 ability, however we also tried to include isolates that have never been reported to inhabit
422 wheat naturally. Interestingly, when the roots of EI seedlings were observed under
423 microscope they were found to be colonised by these fungal isolates successfully. Therefore,
424 our study reports for the first time, the colonization of wheat roots by *Microsphaeropsis*
425 *arundinis*, *Aspergillus ochraceus*, *Aquanectria penicillioides*, *Didymosphaeria variabile.*
426 Among these isolates the best growth promoting activities were exhibited by
427 *Microsphaeropsis arundinis* under both salt and no salt conditions in the glasshouse. This
428 endophyte was isolated from a dicot host but when inoculated on wheat (a monocot), it could
429 not only colonize the new host successfully (Figure 5d) but also promote its growth
430 irrespective of the presence of salt in the growing medium. Amongst the fungal isolates that
431 are known to be natural endophytes of wheat, *Chaetomium globosum* was the best performing
432 isolate. This fungus has been reported to be useful as a bio-control agent against a broad
433 range of pathogens or insect pests[33, 34]. Moreover, its effect on wheat seedlings under drought
434 conditions has also been reported[35]. Therefore, in addition to these reports, based on the
435 results of the current study we suggest that *Chaetomium globosum* may be useful in
436 conferring resistance to biotic stress as well as tolerance to abiotic stress to wheat seedling.
437 The current work represents the first step in the process of identifying candidate endophytes
438 to partner with agricultural plants threatened by saline soils. However, the biochemical and
439 molecular mechanism for conferring salt tolerance to new host needs to be elucidated in
440 future. Furthermore, future studies should be focused on application of potential salt tolerant
441 isolates obtained in this study either alone or in combination to impart salt tolerance in
442 different cereal crops under varied salt concentrations.

443 **CONCLUSION**

444 The amount of salt-affected agricultural land is expected to increase globally in
445 response to climate change. Progress towards increasing crop tolerance to salt through
446 traditional breeding has had limited success, largely because of the genetic complexity of the
447 trait. Endophytes surviving at extreme environmental conditions (dryness, salinity,
448 temperature, heavy metals, etc.) have been found suitable for use in different agricultural
449 practices to combat the effects of such abiotic stress on crop productivity. In the present
450 work, culturable endophytic fungi with different taxonomic affinities were isolated from
451 halophytic plant species. In-vitro studies showed majority of the isolates were found tolerant
452 to high concentration of salt (1.0 M NaCl). When inoculated on seeds of a salt-sensitive
453 wheat genotype (GP#15) these isolates were found to improve germination kinetics of seeds
454 and promote the growth of seedlings under saline conditions.

455 **Acknowledgements**
456 NM acknowledges the Department of Education and Training, Australian Government for
457 providing a fellowship under the Endeavour Research Program, and Murdoch University for
458 hosting the fellowship. NM and MN thank the Indian Council for Agriculture Research and

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459 the Indian Grassland and Fodder Research Institute for granting permission to avail the
460 fellowship program.

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