1 Drought-protecting endophytes shield host plants from sensing the stress

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11 Running title: Endophytes-mediated plant metabolic shift

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14 Originality-Significance Statement

15 In this study we tested our hypothesis that wild plants have retained beneficial endophytes
16 that are not found in related crop plants. We selected two endophytes that were unique to a
17 wheat ancestor plant and showed that they were able to infect bread wheat and confer
18 tolerance to abiotic stress. We further demonstrated that the mechanism by which these
19 endophytes affected the plants was shielding the plants from sensing the stress rather than
20 increasing stress adaptation response. The significance of our findings is that 1) they verify
21 our hypothesis and hence open a new way for quick identification of novel beneficial
22 endophytes, and 2) we suggest a novel mechanism by which the endophytes protect the plants
23 from stress.

24

25

**Summary:**

Endophytes contribute to plant performance, especially under harsh conditions. We therefore hypothesized that wild plants have retained beneficial endophytes, which are less abundant or not present in related crop plants. To test this hypothesis, we selected two endophytes that were uniquely found in sharon goatgrass, an ancestor of wheat, and tested their effect on bread wheat. Both endophytes could infect wheat and improved plants sustainability and performance under water-limiting conditions. To determine how the endophytes modify plant development, we measured parameters of plant growth and physiological status, and performed a comparative metabolomics analysis. Endophytes-treated wheat plants had reduced levels of stress damage markers and reduced accumulation of stress-adaptation metabolites. Metabolomics profiling revealed significant changes in the response to water stress of endophyte-treated plants compared to the metabolic stress response of untreated plants. Our results demonstrate the potential of endophytes from wild plant for improvement of related crops. We show that the beneficial effect of the two endophytes is associated with reduced stress response, suggesting that these endophytes shield the plants from sensing the stress rather than promoting a stress adaptation response.

**Keywords:**

Endophytes, Acremonium, Sarocladium, wheat, wild wheat, metabolic response, stress metabolites, drought tolerance

48 **Introduction**

49 Endophytic fungi form different types of interactions with their hosts, ranging from
50 mutualism to commensalism and parasitism. Mutualistic symbiotic relationships are well
51 known in arbuscular mycorrhiza as well as in several other types of root-inhabiting fungi (van
52 der Heijden *et al.*, 2015; Weiß *et al.*, 2016). Fungal species belonging to these groups have a
53 wide range of proven beneficial functions, including growth enhancement, improved nutrient
54 and water uptake, stress tolerance and pest resistance (Bonfante and Genre, 2010; Baum *et*
55 *al.*, 2015; Gill *et al.*, 2016). Similar beneficial functions are known in Epichloë endophytes,
56 which are a group of sexual (*Epichloë* spp.) and asexual (*Neotyphodium* spp.)
57 clavicipitaceous fungi that inhabit the upper parts of temperate grasses of the subfamily
58 Pooideae (Tanaka *et al.*, 2012; Liu *et al.*, 2017). Studies of the symbiotic relationships in
59 these well-documented systems have shown that the endophytic fungi modify the plant
60 metabolome, for example, the balance of phytohormones, which in turn modifies root
61 development and shoot biomass (Dupont *et al.*, 2015; Wu *et al.*, 2016; Khalid *et al.*, 2018).
62 Other effects include activation of induced systemic resistance (ISR)-like responses, e.g. by
63 the root endophyte *Serendipita indica* (Stein *et al.*, 2008), and production of antifungal and
64 insecticidal alkaloids, for example by *Epichloë* species (Bastias *et al.*, 2017; Florea *et al.*,
65 2017), both of which protect plants from pathogens and insects.

66 Historically, the study of fungal endophytes in grasses focused on clavicipitaceous species in
67 cool-season grasses (Omacini *et al.*, 2012; Schardl *et al.*, 2013). This situation has changed in
68 the past years with the growing interest in endophytes, which led to the discovery of
69 numerous fungal endophytes and characterization of endophytes communities, in a wide
70 range of plant species including grasses (Sánchez Márquez *et al.*, 2012; Chadha *et al.*, 2015).
71 A beneficial function has been assigned in a few cases (Rodriguez *et al.*, 2009; Busby *et al.*,
72 2016; Zheng *et al.*, 2016), however, the vast majority of newly discovered fungal endophytes
73 have not been associated with a specific impact on the host plants and are (still) typed as
74 commensals (Hardoim *et al.*, 2015). Furthermore, only little progress has been made in
75 understanding the mode of action of new beneficial species. Studies of fungal endophytes in
76 wild antecessors of wheat revealed that they include species that could not be found in
77 cultivated wheat, however, they don't contain *Epichloë* endophytes (Ofek-Lalzar *et al.*,
78 2016). This is in line with previous reports that *Epichloë* species in Triticeae were found only
79 in *Hordeum sp.* and *Elymus sp*. (Card *et al.*, 2014). The absence of clavicipitaceous
80 endophytes in wheat and related plant species, suggests that non-clavicipitaceous fungal
81 endophytes might be important in wheat and wheat-related species.

82 In a previous study, we characterized endophyte communities in two wheat ancestral species,
83 *Aegilops sharonensis* (sharon goatgrass), and *Triticum diccocoides* (wild emmer wheat), and
84 compared them with endophytes found in *Triticum aestivum* L (bread wheat) (Ofek-Lalzar *et*
85 *al.*, 2016). Here we describe two endophytes from this study, which were uniquely found in
86 *A. sharonensis*. We show that both endophytes can infect wheat and that they boost wheat
87 growth and drought stress tolerance. We further show that the drought stress-protective effect
88 is associated with delayed and reduced accumulation of stress-related metabolites, suggesting
89 that the endophytes shield the plants from sensing the stress.
90
91 **Results**
92 **Taxonomic identification of the two endophytes from *A. sharonensis***
93 In a previous work, we profiled fungal endophytes found in wheat ancestral species - *A.*
94 *sharonensis* and *T. diccocoides*, and compared them with endophytes found in bread wheat
95 (Ofek-Lalzar *et al.*, 2016). Because cultivated wheat has a relatively narrow genetic
96 background and is grown under optimal conditions, we hypothesized that endophytes that
97 assist wild plants to survive in natural habitats might be absent or less abundant in cultivated
98 wheat. Accordingly, we selected two isolates, #13237 and #14005 that were only found in *A.*
99 *sharonensis* for further analysis. For accurate taxonomic placement, we amplified and
100 sequenced the ORF of *rbp2* and *ef-1α* genes in addition to the ribosomal ITS regionThe ITS
101 (KC987166), *ef-1α* (KC998988) and *rbp2* (KC999024) sequences from isolate #13237
102 showed highest homology (≥99 %) and query coverage (≥97 %), and lowest *e* values (0.0) to
103 *Acremonium sclerotigenum*. In isolate #14005 the ITS (KT878359) and the *ef-1α*
104 (GU189520) sequences showed highest homology (≥99 %) and query coverage (≥97 %), and
105 lowest *e* values (0.0) to *Sarocladium implicatum*, but we found no similarity in the database
106 to the *rbp2* sequence of this isolate. Phylogenetic analysis showed that the fungal isolates
107 #13237 and #14005 had 99% and 94% bootstrap support (500 bootstrap replications) for
108 members of *A. sclerotigenum* and *S. implicatum* respectively, confirming that these isolates
109 are newly identified strains of *A. sclerotigenum* and *S. implicatum* (Fig. 1).
110 **Inoculation of *T. aestivum* with the *A. sharonensis* endophytes**
111 The presence of the fungi was checked by PCR using species-specific primers. Both isolates
112 were found in the roots and stems of all the inoculated plants (100% infection rate),
113 confirming the ability of the isolates to infect wheat (Fig. S1). The inoculated plants did not
114 show any disease symptoms, and compared with control plants we did not observe
115 statistically significant differences in growth parameters, which suggests that the inoculation

116 with any of these two endophytes do not harm or alter the normal growth of the plant. .
117 Moreover, under control conditions, an increase in average dry weight of the roots was
118 observed in *S. implicatum*-infected plants, suggesting a positive effect of the endophytes also
119 under control conditions
120 (Fig. 2F).
121

122 **Plant performance under water limiting conditions**

123 Plants were grown with optimal irrigation for 10 days and then water supply was stopped.
124 Compared with the control plants, the endophyte-infected plants exhibited improved growth
125 parameters; when the control plants reached the wilting point (7-10 days after water supply
126 was stopped), the endophytes-treated plants still retained turgor and green leaves (Fig. 2A).
127 Under water limiting conditions, plants inoculated with *A. sclerotigenum* had higher levels of
128 plant fresh weight, leaf width, leaf area as well as leaf dry weight (Fig. 2B). Notably, for
129 most of the parameters (except root dry weight),*S. implicatum* showed an intermediate
130 phenotype with values that were not statistically significant from either non-inoculated plants
131 or plants inoculated with *A. sclerotigenum.*

132 **Root architecture**

133 Both endophytes improved performance of the plants under water limiting conditions and
134 increased root biomass. To further investigate the effects of the fungi on root development.
135 Overall, the two endophytes modified root architecture, primarily under water limiting
136 conditions (Fig. 3). Compared with plants non inoculated plants, the roots network length
137 under stress situation was increased by 28.09 and 40.78 mm in *S. implicatum* and *A.*
138 *sclerotigenum*-treated plants, respectively (fig 3a). Similarly, roots of the endophytes-treated
139 plants had increased network surface area as well as the network volume compared with non
140 inoculated plants under stress situations (fig 3b and c). On the other hand, under water
141 limiting regime,root number showed no differences between non-inoculated plants and *S.*
142 *implicatum* whereas it was increased in *A. sclerotigenum*-treated plants (fig 3d).

143 **Stress indicators in endophyte-treated plants**

144 The endophytes improved the ability of plants to cope with water deficit, suggested that
145 drought stress responses might be modified. + First, we determined the effect of drought
146 stress conditions on the physiological status of the plants by measurement of relative water
147 content, membrane stability index, and lipid peroxidation. The relative water content was
148 higher in the endophytes-treated plants compared with untreated plants; on average, the
149 relative water content was reduced to 54.3% in the control plants under water limiting

150 conditions, compared with 67.6% and 79.9% in *S. implicatum* and *A. sclerotigenum*-treated
151 plants, respectively (Fig. 4A). The membrane stability index of control plants was reduced by
152 close to 30% under water limiting conditions, compared with only 20% reduction in *S.*
153 *implicatum*-treated plants and less than 10% reduction in *A. sclerotigenum*-treated plants (Fig
154 4B). Similarly, lipid peroxidation, as measured by MDA content, was 10 fold higher under
155 water stress compared with optimal water conditions in control plants, whereas in
156 endophytes-treated plants MDA levels remained low even under severe water stress (Fig.
157 4C). Hence, all of the measured physiological parameters were improved in the endophytes-
158 treated plants under water limiting conditions, indicating that they had reduced damage than
159 the control plants.
160 To determine if the plants' stress response was altered by the endophytes we compared the
161 levels of a number of stress-adaptation metabolites. First, we measured proline levels, which
162 is considered an adaptive response of plants to drought (Bhaskara *et al.*, 2015). Under our
163 experimental conditions, the control and *S. implicatum*-treated plants accumulated high levels
164 of proline under stress conditions, whereas in *A. sclerotigenum*-treated plants proline levels
165 remained low until the end of the experiment (Fig. 5a). Next, we measured the levels of
166 Abscisic acid (ABA) and Jasmonic isoleucine, two plant hormones that accumulate under dry
167 conditions and help plants to adapt to the stress (Sah *et al.*, 2016). ABA amounts were below
168 the detection level in well-watered plants and increased steeply in the control plants to 5.5µg
169 ABA per 1g of dry leaf after 10 days without water (Fig 5b). The endophytes-treated plants
170 also accumulated ABA under water stress conditions, however to lower levels of 3.5µg and
171 2.0 ABA per 1g of dry leaf in *S. implicatum* and *A. sclerotigenum*-treated plants, respectively.
172 Similarly, the levels of jasmonic isoleucine were significantly induced under stress conditions
173 in control plants, but remained low in the endophytes-treated plants (Fig 5c). Phenolic
174 compounds, which are another class of metabolites that accumulate in plants upon water
175 stress (Gharibi *et al.*, 2016) also showed differences between the control and endophyte-
176 treated plants. Levels of caffeic and ferulic acids increased under water stress conditions in
177 the leaves of control plants (Fig. 5d,e), whereas *S. implicatum*-infected plants accumulated
178 moderate levels of both compounds and *A. sclerotigenum*-treated plants did not show a
179 significant difference in the amounts of these metabolites under optimal or drought
180 conditions.
181 Collectively, the analyses of the damage and stress metabolites show that under water
182 limiting conditions the endophytes-treated plants experience less stress damage and

accumulate lower levels of stress-adaptation metabolites. While the reduced stress damages (e.g., water loss and reduced membrane damage) might be expected, the lower levels of stress-adaptation metabolites are surprising in light of the stress-protective role of these compounds (Gharibi *et al.*, 2016; Sah *et al.*, 2016).

**Metabolite profiling of control and infected plants after stress**

To further analyze the metabolic changes induced by the endophytes, we performed a non-targeted metabolomics analysis using UPLC coupled to a Q-TOF MS, and analyzed the data by O-PLS-DA in order to identify possible changes in the metabolic response of control and endophytes-treated plants.

The control and *A. sclerotigenum*-treated plants had different metabolomics profiles under optimal water conditions, but we found a significant metabolic overlap between *A. sclerotigenum*-treated plants under stress conditions and well-watered control plants (Fig. 6a). These results suggest that *A. sclerotigenum* modifies the plant metabolome regardless of the stress and that the fungus moderates the metabolic shift of the plants under stress conditions. Significant differences were found between the effects of the two endophytes on the plant metabolome (Fig. 6b), suggesting that the two fungi might affect plants in different ways.

The data that showed significant differences among endophytes-treated and untreated, and among the optimal and stressed conditions (Kruskal–Wallis test, $P < 0.05$) were used to produce a heat map representation (Fig. 7). The different metabolites were divided into clusters according to their relative amounts in optimal and water limiting conditions, to highlight groups of compounds that are up- or down-regulated during water stress. The heat map analysis of combined positive and negative electrospray ionization ($ESI^-$ and $ESI^+$, respectively) shows that the strongest metabolomics changes are produced by the drought stress, but significant differences were observed between the control and endophytes-treated plants. Among the metabolites that were shared between the control and *A. sclerotigenum*-treated plants, 61.3% were strongly down-regulated in the control plants under water stress, whereas only a subset of these compounds was downregulated in the *A. sclerotigenum*-treated plants (Fig. 7 top, clusters 1 to 4). Similarly, 41.5% of the metabolites shared by the control and *S. implicatum*-treated plants were strongly down-regulated in the non-inoculated plants under water stress plants but not in the *S. implicatum*-treated plants (Fig 7 bottom, clusters 5 to 7). Additionally, the *A. sclerotigenum* and *S. implicatum*-treated plants showed down-regulation of 28.45% (Fig 7 top clusters 5 to 7) and 9.8% (Fig 7 bottom cluster 1) respectively, of metabolites that were not detected in any other treatment. Taken together, these results indicate that the presence of endophytes in the plant modulates the accumulation

217 of metabolites, which may be related to the expression or repression of defensive mechanisms
218 against stress.
219 The lists of metabolites obtained from the metabolomics analysis were further analyzed in the
220 context of plant pathway databases by comparison of the exact mass of the altered
221 compounds with several online databases using the Mar-Vis Pathway tool. This analysis
222 showed that both endophytes induced changes in the 2-Oxocarboxylic acid metabolism
223 pathway, amino acids biosynthesis (in particular arginine and tryptophan), and the pathway of
224 aromatic compounds degradation (Table S1). These results hint to a possible change in amino
225 acids balance and metabolism, as well as highlight plant metabolic pathways that might be
226 modified in endophyte-treated plants under stress conditions.
227
228 **Discussion**
229 . Previous studies about the microbiome of wheat antecessors showed strong differences in
230 the number and composition of fungal endophytic communities(Ofek-Lalzar *et al.*, 2016).
231 Moreover, the absence of clavicipitaceous endophytes in Triticum species suggests an
232 important role of non-clavicipitaceous endophytes in plant performance. To test this
233 possibility, we selected two strains that were previously isolated from *A. sharonensis,* and
234 tested their ability to infect and affect wheat plants. The two isolates belong to the genera
235 *Acremonium* and *Sarocladium* and BLAST analysis (Altschul *et al.*, 1990)showed that they
236 represent new strains of *Acremonium sclerotigenum* and *Sarocladium implicatum*.
237 Interestingly, the genera *Acremonium* and *Sarocladium* are closely related to the genus
238 *Epichloë,* and were previously classified as a single genus together with *Epichloë* (Glenn *et*
239 *al.*, 1996; Giraldo *et al.*, 2015).
240 Following seed inoculation, both endophytes could infect wheat seedlings, as well as maize,
241 barley, tomato, and beans, and were detected in roots, crown and stems of the plants (not
242 shown). Hence, although the new strains originated from *A. sharonensis*, they could also
243 penetrate and exist as endophytes in other plant species, including wheat. The ability of the
244 two endophytes to infect various plant species, place them in class 2 endophytes (Rodriguez
245 *et al.*, 2009) together with a large group of endophytes belonging to different taxonomic
246 groups, which are capable of horizontal transfer and are found in a wide range of plant
247 species (Saikkonen *et al.*, 2004). This is in sharp contrast to *Epichloë* species, which are
248 primarily vertically transmitted and are host-specific. These differences between the more
249 generalist endophytes and *Epichloë* endophytes hint to possible differences also in
250 endophytic lifestyle and in the effect on the host, of fungi belonging to either class.

251 To characterize the possible effect of the new endophytes on plants, we compared growth
252 characteristics of control and endophytes-infected wheat plants. Both species had a positive
253 effect on plant growth under water limiting conditions, with *A. sclerotigenum* always
254 showing superiority over *S. implicatum.* Similar positive effects on growth and stress
255 tolerance are well known in *Epichloë* endophytes (Rodriguez *et al.*, 2009; Nagabhyru *et al.*,
256 2013; Vázquez-de-Aldana *et al.*, 2013; Kauppinen *et al.*, 2016), as well as in non-
257 clavicipitaceous endophytes from various host plants including wheat and barley (Hubbard *et*
258 *al.*, 2014; Murphy *et al.*, 2014, 2015; Hosseini *et al.*, 2017; Taghinasab *et al.*, 2018). The
259 improved plant growth under stress conditions were accompanied by reduced damage to the
260 plants, as indicated by increased relative water content and membrane stability index, and
261 reduced levels of lipid peroxidation. These results are in agreement with previous reports,
262 showing that leaves of fungal inoculated plants are less affected by stress conditions and
263 maintain higher water content compared with untreated plants (Morse *et al.*, 2002; Swarthout
264 *et al.*, 2009).
265 The increased water levels in leaves of endophyte-treated plants could be related to the
266 improved ability of the plants to absorb water from the soil, for example, due to altered root
267 architecture, as was demonstrated in *E. coenophiala*-infected tall fescue plants (Hosseini *et*
268 *al.*, 2016). Indeed, both endophytes increased root biomass and modified root architecture,
269 suggesting improved ability to absorb water.
270 Surprisingly, however, the endophyte-infected plants had drastically lower levels of stress
271 response markers. For example, proline levels remained very low and levels of phenolic
272 compounds were only slightly elevated in endophyte-infected plants under water limiting
273 conditions, compared with significant increase of these stress metabolites in control plants.
274 These results suggest that infection of plants with either one of the two new endophytes
275 protected the plants from sensing the stress and therefore delayed the stress response of the
276 plants, rather than increasing the plants stress adjustments responses. Similar results were
277 previously reported in lettuce, soybean and rice, where plants inoculated with different
278 endophytes showed improved performance under saline conditions along with lower ABA
279 levels (Khan *et al.*, 2011, 2012).
280 While for most of the measured parameters the effect of *A. sclerotigenum* was stronger than
281 the effect of the *S. implicatum* isolate, both endophytes modified the development of plants
282 such that the plants remained in a better physiological state under water stress conditions, and
283 therefore were less sensitive to external stress. More significant differences were observed in
284 effect of the two endophytes on metabolic shifts of the plants under normal and stress

285 conditions. The metabolic behavior of plants under optimal and stress conditions changed
286 only slightly in *A. sclerotigenum*-infected plants, whereas the *S. implicatum*-infected plants
287 showed a significantly different metabolic behavior compared with untreated control and
288 stressed plants. The differences in the effect of the two fungi on metabolic shifts of the plants
289 indicate that while both endophytes boost plant tolerance to stress, they might differ in their
290 specific effects. Interestingly, the clusters of compounds that altered only in the presence of
291 both endophytes and drought stress mainly include metabolites that were obtained in the
292 negative ionization mode.
293 Analysis of the most significantly altered metabolites highlighted the 2-oxocarboxilic acid
294 metabolisms as well as several different amino acid related pathways. The 2-oxocarboxilic
295 acid pathway is associated to the biosynthesis of different amino acids, mainly asparagine and
296 glutamate, which are involved in resistance against abiotic stress, through the production of
297 2-oxoacids (O'Leary *et al.*, 2011). Glutamate is directly associated with abiotic plant
298 response because it is used as a precursor for production of compatible osmolytes including
299 proline and polyamine (Roberts, 2005; Yamamoto *et al.*, 2015). Additionally, amino acids
300 such as tryptophan and phenylalanine accumulate in maize and wheat under drought stress
301 (Bowne *et al.*, 2012; Witt *et al.*, 2012). Tryptophan is a target of oxidation and its presence as
302 a free amino acid in the plant can act as a buffer, protecting the cells against reactive oxygen
303 species (ROS) damage.
304 In conclusion, under our experimental conditions, plants inoculated with either *A.*
305 *sclerotigenum* or *S. implicatum* had reduced levels of stress responses compared with
306 untreated plants. Therefore, our results indicate that under water stress conditions, the
307 endophytes don't boost the stress response of the plants, but rather improve the physiological
308 status of the plants thereby allowing improved performance of the plants under stress
309 conditions. In this way, the addition of endophytes to the crops can be used to add new traits
310 of resistance against stress. However,more studies will be necessary in order to ascertain the
311 mecanisms induced by the endophytes to improve the stress resistance in plants.

312 **Materials and methods**

313 **Fungi**

314 **Selection of isolates:** We used two pure fungal strains that were isolated from stems of
315 Sharon goatgrass (*Aegilops sharonensis*), a wheat ancestor that grows exclusively on the
316 Mediterranean coastline in Israel, between Lat 32º 54' 31.5720" Lon 35º 05' 18.8939" and Lat
317 31º 23' 53.2068" Lon 34º 18' 16.4259" (Millet *et al.*, 2006). Isolate #13237 was obtained
318 from F1 plants of seeds that were collected at Palmachim site, Lat 31º 55' 57.2844" Lon 34º

319 43' 44.1516", isolate #14005 was obtained from F1 plants of seeds that were collected at
320 Netanya site, Lat 32° 25' 3" Lon 34° 53' 18.887". The two isolates were selected based on
321 high relative abundance in *A. sharonensis* but not in cultivated wheat, and according to ITS
322 sequences, which classified them to the genera *Acremonium* (#13237) and *Sarocladium*
323 (#14005), both of which include beneficial endophytic species (Breen, 1994; Tian *et al.*,
324 2014). For more accurate taxonomic identification, genomic DNA was isolated from fresh
325 mycelia using the CTAB method (Tamari *et al.*, 2013). The ITS region, and the open reading
326 frames (ORF) of elongation factor 1α (*EF-1α*) and second large subunit of RNA polymerase
327 II (*RPB2*) genes were amplified by PCR using conserved primers as described by Sung et al.
328 (Sung *et al.*, 2007) and Berbee et al. (Berbee *et al.*, 1999), respectively. PCR fragments were
329 cleaned, sequenced and the sequences were searched by BLAST against the NCBI
330 nonredundant fungal database.
331 **Culturing and spore production.** Fungi were routinely cultured on potato dextrose agar
332 (PDA) supplemented with 100 µg/ml ampicillin and 50 µg/ml kanamycin and maintained in a
333 growth chamber at 25°C with continuous fluorescent light. For spore production, mycelia
334 were obtained from 7-day old cultures and used to inoculate flasks containing 150ml of
335 potato dextrose broth (PDB). The cultures were incubated for five days in a growth chamber
336 at 25°C with continuous light and agitation at 180 rpm. Spores were collected by filtration of
337 the cultures through two layers of miracloth (Calbiochem), the spores were counted using a
338 hemocytometer, and the density was adjusted with PDB to $10^6$ spores/ml.
339 **Plants**
340 **Seed germination**. Wheat seeds (*Triticum aestivum* cv. Galil) were surface-sterilized by
341 soaking for 1 minute in 70% ethanol followed by 3 minutes in 3% bleach and then rinsed
342 three times with sterile distilled water. Surface sterilized seeds were placed in a Petri dish
343 with wet filter paper for vernalization at 5ºC. After 24h the seeds were moved to a growth
344 chamber for germination at 25ºC with 12/12 light-dark regime for 72h.
345 **Treatment with endophytes**. Spores were suspended in PDB and roots of germinated
346 seedlings were soaked for two hours the spore suspension. Control seeds were similarly
347 treated by soaking for two hours in sterile PDB. The germinated seeds were planted in 500ml
348 pots with 400g of autoclaved loam soil for water limiting conditions experiments.
349 **Water stress:** Plants were maintained in a greenhouse with temperature ranging between
350 18ºC and 24ºC and light-dark regime. Each treatment included five pots with four seeds per
351 pot. Plants were fertigated with Sheffer fertilizer (7-3-7, Fertilizers, Israel), or with half-
352 strength Hoagland nutrient solution every second day. Water stress was applied by quitting

353 water supply 10 days after planting and the plants were harvested 10 days from the last day of
354 irrigation, at which time the un-treated (control) wheat plants reached a wilting point.

355 **PCR analysis**

356 **Design of species-specific PCR primers**. Specific primers for detection of each of the
357 isolates were designed according to the *rbp2* gene sequences. The sequences were aligned
358 with *rbp2* homologs from fungi representing a wide range of taxonomic groups, and regions
359 with sequences that were found unique to the tested isolate were selected. Species-specific
360 primers were designed for identification of each of the two strain: RPD1M forward and
361 reverse for identification of isolate #13237, and RPD4M forward and reverse for
362 identification of isolate #14005 (Table S2).

363 **PCR reactions**. Specificity of the primers was tested against a mock community containing
364 DNA from *Alternaria malorum, Stemphylium* sp., *Alternaria infectoria*, and both endophytes.
365 For the preparation of the mock community, DNA was extracted from each of the fungal
366 species using the CTAB method (Tamari *et al.*, 2013), 20ng DNA samples from each species
367 were mixed, and then different amount of DNA of the two endophytes were added,at a ratio
368 of 1:10, 1:10 and 1:1000 (supplementary figure 2). PCR reactions (25µl) contained 0.7µl of
369 each primer (10pmol), 12.5µl Hay-taq ready mix PCR reaction mix (Hy Laboratories, Israel),
370 8.6µl DDW and 2.5µl of template DNA (minimum 100ng). PCR cycling conditions for these
371 reactions were: initial denaturation 95°C for 5 min, followed by 35 cycles of 95°C for 30
372 seconds, 63°C for 30 seconds, 72°C for 45 seconds, and final elongation at 72°C for 10
373 minutes.

374 **Measurement of effects of endophytes on plants**

375 **Morphological parameters**. Shoot length and biomass, leaf width, and root length, biomass,
376 and architecture were measured. The length of the single longest root was used as a measure
377 of root length. The height of the plant, as measured from the soil to the top leaf, was used as a
378 measure of shoot length. Plant fresh weight was obtained by measuring the weight of aereal
379 parts and roots. The dry weight of all above ground parts was used as a measure of shoot
380 biomass, and the dry weight of all underground parts was used as a measure of root biomass.
381 The width of the second blade was measured with ImageJ software as described by Juneau
382 and Tarasoff (Juneau and Tarasoff, 2012) and used as a measure of leaf width.

383 **Physiological parameters.** Relative water content, membrane stability index, lipid
384 peroxidation, and proline levels were measured. Relative water content was measured as
385 described by Pask et al. (Pask *et al.*, 2012). Fresh leaf samples were weighed to obtain the
386 fresh weight (FW), and then placed in water, chilled overnight, weighed again to obtain the

387 turgid weight (TW), and then dried in an oven and weighed again to obtain the dry weight
388 (DW). The relative water content was determined as follows:
389 Leaf relative water content (%) = [(FW-DW) / (TW-DW)] × 100
390 Membrane stability index was determined by recording the electrical conductivity of leaf
391 leachates as described by Sairram et al (Sairam *et al.*, 2002). Leaf samples were cut in discs
392 and placed in test tubes each containing 10ml of sterile distilled water and remained at room
393 temperature for 22h. After this time, electric conductivity was measured using a conductivity
394 meter to obtain the C1. Then, the tubes were incubated at 90ºC for 2 hours and the electric
395 conductivity was measured to obtain C2. Membrane stability was calculated as 1-(C1/C2)
396 and expressed as a percent.
397 To analyze root architecture, pre-germinated wheat seeds were grown in paper-based growth
398 method for non-destructive development as previously described by Planchamp et al.
399 (Planchamp *et al.*, 2013). The plants were maintained in a growth chamber with a light period
400 of 14/10 h light-dark regime (400 µmol photosynthetically active radiation $m^{-2}$ $s^{-1}$), at a
401 temperature of 26/18°C (light/dark, respectively) and 60% humidity. Plants were grown one
402 week with a constant supply of water amended with Sheffer fertilizer. Pictures of the roots
403 were analyzed with GIAroots software framework (Galkovskyi *et al.*, 2012).
404 Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation using
405 the thiobarbituric acid method as described by Madhava Rao and Sresty (Madhava & Sresty,
406 2000). For MDA extraction, 0.5g of root samples were homogenized with 2.5ml of 0.1%
407 trichloroacetic acid (TCA). The homogenate was centrifuged for 10 min at 10,000*g*, the
408 supernatant was removed and mixed at a 1:4 ratios with 20% TCA containing 0.5%
409 thiobarbituric acid. The mixture was heated at 95°C for 30 min, chilled on ice, and then
410 centrifuged for 15 min at 10,000*g*. The supernatant was removed to a fresh tube, and
411 absorbance at 532nm was measured and corrected for non-specific turbidity by subtracting
412 the absorbance at 600nm. The concentration of MDA was calculated using an extinction
413 coefficient of 155$mM^{-1}$ $cm^{-1}$.
414 Free proline content was measured according to Bates et al. (Bates *et al.*, 1973). Root
415 samples (0.5g) from each group were homogenized in 3% (w/v) sulphosalicylic acid and the
416 homogenate was centrifuged at 4000*g* for 40 min. One ml of supernatant was mixed with 1ml
417 of 2.5% acid ninhydrin and 1ml of glacial acetic acid, the mixture was incubated at 100 °C
418 for 1h and then chilled on ice to stop the reaction. The solution was extracted with 3ml of
419 toluene, the toluene fraction was aspired and the absorbance of the organic phase was

420 measured at 520nm. Proline concentration was determined using a calibration curve and
421 expressed as µmol proline $g^{-1}$ FW.

422 **Chromatographic analysis**

423 For analysis of hormones and phenolic compounds, fresh plant material was frozen in liquid
424 nitrogen and lyophilized. Dry tissue (50µg) was homogenized in 1ml of ultrapure water and a
425 mixture of internal standards was added to each sample (100ng of [$^2H_6$]-ABA, 100ng each of
426 prostaglandin B1, dihydrojasmonic acid, and propylparaben). The mixture was centrifuged at
427 4,000*g* for 40 min and the supernatant was acidified with acetic acid to pH 2.7. Samples were
428 mixed with an equal volume of diethyl ether, centrifuged at 4000*g* for 1 min and the organic
429 phase was recovered and dried in a speed-vac. The dry residue was suspended in 1ml of
430 MeOH/water (10/90%), and 20µl aliquots were injected into a Waters AQUITY UPLC
431 system (Milford, MA, U.S.A.) with nucleosil ODS reversed-phase column (100 by 2mm, i.d.
432 5µm, Scharlab, Barcelona, Spain). The chromatographic system was interfaced to a Quatro
433 LC (quadrupole-hexapolequadrupole) mass spectrometer (Micromass, Manchester, U.K.).

434 **LC-ESI full scan mass spectrometry (Q-TOF) and bioinformatics analysis**

435 Fresh plant material was frozen in liquid nitrogen and lyophilized and 50µg of the dry tissue
436 was homogenized in 1ml of ultrapure water. The mixture was centrifuged at 4,000*g* for 40
437 min, the supernatant was recovered and 20µl aliquots were injected into a UPLC (Waters,
438 Mildford, MA, USA) coupled to a quadrupole-time of flight mass spectrometer (QTOF
439 Premier) through an electrospray ionization source. The LC was developed for 25min in a
440 common C18 column using a standard variable $H_2O$:MeOH gradient and mass detection was
441 performed using 25v of cone energy. The drying gas and the nebulizing gas was nitrogen.
442 The desolvation gas flow was set to approximately 600l/h, and the cone gas flow was set to
443 60l/h. A cone voltage of 20v and a capillary voltage of 3.3kv were used in the negative
444 ionization mode. The nitrogen desolvation temperature was set at 350°C, and the source
445 temperature was set at 120°C. The instrument was calibrated in the m/z 50–1000 range with a
446 1/1 mixture of 0.01M NaOH/1% HCOOH ten-fold diluted with acetonitrile/water (80/20,
447 v/v). Raw data obtained from MASSLYNX software were transformed to CDF format using
448 the DataBridge program provided with the MASSLYNX software. The CDF data were
449 processed with R for statistical computing using the XCMS package for relative
450 quantification(Smith *et al.*, 2006) (Smith et al., 2006). To determine a global behavior of the
451 signals, Orthogonal Partial Least squares discriminant analyses (O-PLS-DA) plots were
452 generated using the DEVIUM package for R, which is a multivariate statistical method that

453 can differentiate between classes in highly complex data sets. For the heat map construction,
454 clustering of metabolite and analysis of results MarVis suite were used
455 (http://marvis.gobics.de/) (Kaever *et al.*, 2012).

456 **Statistical analyses**

457 Phylogeny analysis was performed with Mega software version 7. Statistical analyses were
458 performed using Statgraphics Centurion XVI (Statpoint Technologies, Warrenton, VA,
459 USA). Data were submitted to 2- way ANOVA analysis for population groups that follow a
460 normal distribution and the means were separated using Fisher's least significant difference
461 (LSD) at 95%.

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465

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637
638

639 **Figure legends**

640 **Figure 1. Taxonomic placement of isolates 13237 and 14005**. Combined sequences of the
641 *ITS* region and *ef1- α* gene of the two isolates were used to BLAST search the NCBI
642 database. A maximum likelihood phylogenetic tree of the most closely related sequences was
643 constructed by neighbor-joining method. Number on the branches represent support values
644 from 500 ML bootstrap replicates. The scale bar on the rooted tree indicates a 0.02
645 substitution per nucleotide position.

646

647 **Figure 2. Effect of the two endophytes on plant development under optimal and water**
648 **limiting conditions**. Endophyte-treated and untreated wheat seeds were planted in loam soil
649 and grown in a greenhouse. Water supply was stopped after 10 days, and samples were
650 collected when the control plants reached wilting point. A. picture of plants 10 days after
651 stopping water supply. B. Plant growth parameters. Values are means of three independent
652 experiment ± standard error (SE n=20). Different letters indicate statistically significant
653 differences for each treatment (optimal or stress) at $P \leq 0.05$ according to Fisher's protected
654 LSD test.

655

656 **Figure 3. Effect of the two endophytes on root development under optimal and water**
657 **limiting conditions**. Germinated seeds were inoculated and mounted in germination paper.
658 The seedlings were maintained with nutrient solution for seven days after which water supply
659 was stopped. After 14 days, the roots were photographed, the number of roots per plant was
660 determined and root architecture was analyzed. Values are means of three replicates ±
661 standard error (SE n=20). Different letters indicate statistically significant differences for
662 each treatment (optimal or stress) at $P \leq 0.05$ according to Fisher's protected LSD test.

663

664 **Figure 4. Effect of the two endophytes on accumulation of stress damage markers**.
665 Endophyte-treated and untreated wheat seeds were planted in loam soil and grown in a
666 greenhouse. Water supply was stopped after 10 days, and samples were collected when the
667 control plants reached wilting point. Values are means of three replicates ± standard error (SE
668 n=20). Different letters indicate statistically significant differences for each treatment
669 (optimal or stress) at $P \leq 0.05$ according to Fisher's protected LSD test.

670

671

672 **Figure 5. Effect of the two endophytes on accumulation of stress-adaptation metabolites**
673 **in wheat leaves**. Endophyte-treated and untreated wheat seeds were planted in loam soil and
674 grown in a greenhouse. Water supply was stopped after 10 days, and samples were collected
675 when the control plants reached wilting point. Values are means of three replicates ± standard
676 error (SE n=20). Different letters indicate statistically significant differences for each
677 treatment (optimal or stress) at $P \leq 0.05$ according to Fisher's protected LSD test
678
679 **Figure 6**. **Score plots from the results of orthogonal partial least squares discriminant**
680 **analyses (O-PLS-DA).** Left: *Acremonium sclerotigenum* inoculated plants, right: *S.*
681 *implicatum* inoculated plants. Endophyte-treated and untreated wheat seeds were planted in
682 loam soil and grown in a greenhouse. Water supply was stopped after 10 days, and samples
683 were collected when the control plants reached wilting point. Leaf material from 20
684 individual plants was pooled for each treatment combination. Data points represent three
685 independent experiments injected randomly into the UPLC-QTOF MS. Ellipses in the score
686 plot of this figure illustrate the 95% confidence regions of the corresponding groups.
687
688 **Figure 7. Heat map analysis for combined $ESI^+$ and $ESI^-$ signals obtained from a non-**
689 **targeted analysis by UPLC-QTOF MS.** Results show the most significant metabolomics
690 categories that were changed by presence of the endophytes under optimal and water limiting
691 conditions. Top: *A. sclerotigenum*-treated plants, bottom: *S. implicatum*-treated plants. Leaf
692 material from 20 individual plants was pooled for each treatment combination. Figure
693 represents three independent experiments injected randomly into the UPLC-QTOF MS.
694 Signals corresponding to different treatments were compared using the non-parametric
695 Kruskal–Wallis test, and only data with a $P < 0.05$ between groups were used for subsequent
696 processing.
697
698 **Figure S1. Detection of isolates 13237 and 14005 in inoculated wheat plants**. Inoculated
699 and uninoculated wheat seedlings were sown in pots and grown in a greenhouse. At two
700 leaves stage the plants were sampled, DNA was extracted using CTAB method and the
701 presences of the isolated was tested by PCR. A: PCR reactions tested with A. sclerotigenum
702 specific primers, lanes 1-4 are un-inoculated plants, lanes 5-10 are 13237 inoculated plants
703 and lanes 11,12 are controls, positive with *A. sclerotigenum* DNA and no added DNA,
704 respectively. A: PCR reactions tested with S. implicatum specific primers, lanes 1-4 are un-

705 inoculated plants, lanes 5-10 are 14005 inoculated plants and lanes 11,12 are controls,
706 positive with *S. implicatum* DNA and no added DNA, respectively.
707
708 Figure S2. Specifitiy of primers: Gel electrophoresis results of mock community tests. In
709 each gel lanes 1-2 are positive and negative mock communities respectively, lanes 3-4 are
710 mock samples diluted 1:10, lanes 5-6 are mock samples diluted 1:1000, lane 7 is positive
711 control and lane 8 is negative control without added DNA. A. 13237 PCR markers (RPD1M).
712 B. 14005 PCR markers (RPD4M).