

1 **Bacterial biofilm formation on soil fungi: a widespread ability under controls**

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23

24 **Abstract**

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26 In natural environments, bacteria preferentially live in biofilms that they build on abiotic surfaces but also  
27 on living tissues. Although fungi form extensive networks of hyphae within soils and thus could provide  
28 immense surfaces for bacteria to build biofilms and to proliferate, the extent on such phenomenon and  
29 the consequences for the fitness of both microorganisms is poorly known in soils. Here, we analyzed  
30 the process of formation of biofilms by various bacteria on hyphae of soil fungi in an *in vitro* setting using  
31 confocal and electron microscopy. We showed that the ability to form biofilms on fungal hyphae is  
32 widely shared among soil bacteria. In contrast, some fungi, mainly belonging to the Ascomycete class,  
33 did not allow for the formation of bacterial biofilms on their surfaces. The formation of biofilms was also  
34 strongly modulated by the presence of tree roots and by the development of the ectomycorrhizal  
35 symbiosis, suggesting that biofilm formation does not occur randomly in soil but that it is highly  
36 regulated by several biotic factors. Finally, our study led to the unexpected finding that networks of  
37 filaments made of extracellular DNA were used to build the skeleton of biofilms by a large array of  
38 bacteria.

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43 **Keywords:** biofilm, eDNA, ectomycorrhizal symbiosis, soil fungi, fungal/bacterial interactions

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45 **Statistics**

46 **Abstract.** 200 words (200 max)

47 **Main text.** 4332 words (5000 max)

48 **Figures & Tables.** 2 Tables, 6 figures

49 **Supplemental Figures & Tables.** 4 figures, 1 table.

50 **References.** 56 (100 max)

## 51 Introduction

52           Among the myriad of organisms that live in forest soils, bacteria and fungi largely exceed their  
53 counterparts in terms of abundance and diversity (Nazir *et al.*, 2010). Both highly contribute to the  
54 decomposition of soil organic matter and to the nutrient cycling and thus have a key role in the  
55 modulation of soil fertility and productivity (Rousk and Bengtson, 2014; Lindahl and Tunlid, 2015). In  
56 addition, some mutualistic fungi called mycorrhizal fungi, act as providers of carbon sources to the soil  
57 and of nutrients to the trees through the symbiosis they establish with roots (Smith and Read, 2008).  
58 In soil, many bacteria and fungi often occupy a shared microhabitat and there, many bacteria often  
59 colonize the surface of fungal hyphae, also called “hyphosphere” (Frey-Klett *et al.*, 2011). Bacteria are  
60 thought to gain two main benefits from this association. First, the hyphosphere provide a nutritional  
61 source for bacteria that either consume nutrients released directly or indirectly by hyphae, or directly  
62 prey on fungi (Leveau and Preston, 2008; Hover *et al.*, 2016). Second, fungal hyphae can serve as  
63 vectors for bacteria to travel across the soil and to reach otherwise inaccessible nutrient sources (Nazir  
64 *et al.*, 2010). These so called “hyphal highways”, can be followed by bacteria that swim along the water  
65 film that covers the hyphae, or by bacteria that settle at the tip of the growing hyphae (Otto *et al.*, 2016;  
66 Warmink and van Elsas, 2009). Conversely, some fungi can benefit from the metabolic activity of their  
67 associated bacteria (Li *et al.*, 2016), gain protection against stresses (Nazir *et al.*, 2014) or even “farm”  
68 bacteria to later use them as a source of nutrients (Pion *et al.*, 2013). However, this close interaction  
69 between fungi and bacteria can also be detrimental to the fungi and a number of them produces  
70 defensins to prevent the bacterial colonization of their hyphae (Essig *et al.*, 2014).

71           Bacteria can establish in the hyphosphere in three states: as free-living cells, as attached single  
72 cells or as organized biofilms. Biofilms arise through the aggregation of bacterial cells and their  
73 embedding into a self-produced matrix of extra polymeric substances (Flemming *et al.*, 2016). Life as a  
74 biofilm has the double advantages to increase the bacterial resistance against biotic and abiotic  
75 stresses, and to permit the organization of cells into functional sub-communities. As a consequence, a  
76 large number of bacterial species have developed the ability to build biofilms on hydrated abiotic  
77 surfaces (e.g. water pipes, medical devices...) but also on living tissues (e.g. epithelial cells, root  
78 surfaces...). Fungal hyphae can also support bacterial biofilms and *in vitro* formation of bacterial  
79 biofilms on hyphae of some soil Ascomycetes, Basidiomycetes and Zygomycetes has been reported  
80 (Scheublin *et al.*, 2010; Burmølle *et al.*, 2012; Hover *et al.*, 2016; Nazir *et al.*, 2014). Yet, while soil fungi  
81 offer relatively close habitats to bacteria, different behaviors exist between soil fungal species. Since  
82 bacterial biofilms are frequent on sporocarps of ectomycorrhizal (ECM) fungi but rare on saprotrophic  
83 ones, it has been proposed that the trophic status (i.e. symbiotic vs saprotrophic) of the fungi drives the  
84 interactions (de Carvalho *et al.*, 2015). As being in competition for carbon sources, saprophyte would be

85 more intolerant to bacteria than mycorrhizal fungi that get their carbon from their plant partner. However,  
86 this hypothesis has not been tested on hyphae yet. Conversely, differences among bacteria in their  
87 ability to form biofilms on hyphae have been pointed in the arbuscular mycorrhizal (AM) fungi  
88 *Rhizophagus intraradices* and *Glomus* sp. (Toljander *et al.*, 2006; Scheublin *et al.*, 2010) suggesting a  
89 potential fine-tuned interaction between these fungi and bacteria during the process of biofilm formation.  
90 Specific bacterial communities also preferentially associate with ECM fungi (Frey-Klett *et al.*, 2005;  
91 Warmink *et al.*, 2009). Among the ECM associated bacteria, Mycorrhiza Helper Bacteria (MHB) have  
92 received a lot of attention because of their ability to stimulate mycorrhiza formation and functioning  
93 (Deveau and Labbé, 2017). Some of them have been shown to form biofilm-like structure on ECM fungi  
94 although the extent of such interactions is unknown (Frey-Klett *et al.*, 2007). Because ECM fungi  
95 colonize very large volumes of soil, they could provide large surfaces for bacteria to establish as  
96 biofilms. Altogether, these data suggest that biofilm formation on fungal hyphae could be an important  
97 phenomenon in soil. However, we have little information on the frequency of such event, its level of  
98 specificity among microorganisms and the biotic and abiotic parameters that drive the fate of the  
99 interaction. Such knowledge is key to predict how soil fungi and bacteria behave in soils.

100 To fill this gap, we characterized in depth the biofilms formed by several MHBs and other soil  
101 bacteria on hyphae of the model ECM fungus *Laccaria bicolor* S238N (Martin *et al.*, 2008) using an *in*  
102 *vitro* set up (Miquel Guennoc *et al* 2016). We then evaluated the impact of the root system and of the  
103 ectomycorrhizal symbiosis on the fate of the interaction. Lastly, we tested the impact of the trophic  
104 status and the taxonomic origin of soil fungi on the establishment of biofilms. Our data suggest that  
105 biofilm formation on hyphae does not occur randomly but then it is regulated by several biotic factors. In  
106 addition, the detailed study of the structure of the bacterial biofilms revealed an unexpected feature that  
107 is likely to be widespread: the use of extracellular DNA filaments to build the skeleton of biofilms.

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## 111 **Material and methods**

### 112 *Microbial strains and culture conditions*

113 All the strains used in this study are listed in Table 1. GFP-tagged versions of *Pseudomonas*  
114 *fluorescens* BBc6 (Deveau *et al.*, 2010), *Burkholderia ginsengisoli* E3BF7\_7 and *Dyella* sp. E3BF9\_7  
115 were used to facilitate imaging. None of the other microbial strains used in this study constitutively  
116 expressed a fluorescent protein. Bacterial strains were maintained at  $-80^{\circ}\text{C}$  in Luria-Bertani (LB)  
117 medium with 30% glycerol and were first grown on 10% tryptic soy agar (TSA)-plates for 24h (3 g.l<sup>-1</sup>  
118 tryptic soy broth from Difco and 15 g.l<sup>-1</sup> agar) at  $28^{\circ}\text{C}$ . Then, for each strain, 2 to 3 individual bacterial  
119 colonies were collected from TSA cultures to inoculate 25 ml of liquid LB medium and incubated at

120 28°C and 150 rpm until late exponential growth before their use for biofilms formation. Fungal cultures  
121 were maintained on P5 medium then transferred to P20 agar plates covered with EDTA pre-treated  
122 cellophane membranes as described by Miquel Guennoc *et al.* (2016), except for *Tuber melanosporum*  
123 that was always kept on P20 medium.

124

#### 125 *In vitro* biofilm formation on fungal hyphae and glass fibers

126 The method described step-by-step in Miquel Guennoc *et al.* (2016) was used. Briefly, bacterial cultures  
127 in late exponential growth phases were spin down and washed once in potassium phosphate buffer  
128 (PPB; KH<sub>2</sub>PO<sub>4</sub> 25 g.l<sup>-1</sup>, K<sub>2</sub>HPO<sub>4</sub> 2.78 g.l<sup>-1</sup>, pH 5.8). Bacterial pellets were suspended in PPB and the cell  
129 density was adjusted to 10<sup>9</sup> cfu.ml<sup>-1</sup> to prepare the bacterial inoculum. Five ml of this bacterial  
130 suspension was added to each well of 6 well-microplates. Then, using sterile tweezers, 1-cm diameter  
131 fungal colonies grown on P20-agar plates were added to each well of the microplates. The cellophane  
132 membranes on which they were growing were kept to avoid harming fungal hyphae during the transfer.  
133 Plates were gently shaken for 1 min to allow the fungus to unstick from the cellophane sheets and  
134 cellophane sheets were removed from each well. These resulting microplates with the bacterial and  
135 fungal inocula were incubated at 20°C and 60 rpm for 16hrs, except if otherwise stated. To test for  
136 biofilm formation on dead fungal colonies, 1-cm diameter growing fungal colonies were dipped into  
137 either 3% paraformaldehyde solution for an hour or progressive ethanol baths for 3 min each (20%,  
138 50%, 70%, 100 %), then washed three times with PPB to remove traces of paraformaldehyde or  
139 ethanol. Dead colonies were then immediately used to test for biofilm formation using same protocol  
140 as above. To test the formation of bacterial biofilm on glass fibers, fungal colonies were replaced by 10-  
141 µm diameter sterile glass fibers. For each treatment, two independent assays in triplicate were  
142 analyzed.

143

#### 144 *Sample preparation for confocal and electron microscopy imaging*

145 Samples for imaging were prepared following the methodology described in Miquel Guennoc *et al.*  
146 (2016). Briefly, fungal colonies or glass fibers were transferred to a new microplate. Then, samples were  
147 rinsed with NaCl (17 g.l<sup>-1</sup>) and with PPB to detach planktonic and electrostatically attached bacterial  
148 cells (Toljander *et al.*, 2006).

149 For confocal imaging, fungal colonies were cut in half with a razor blade, stained then mounted on slide  
150 with Fluoromount-G anti-fading (Fisher Scientific). Fungal hyphae were stained with 10 µg.ml<sup>-1</sup> Wheat  
151 Germ Agglutinin coupled to Alexa Fluor 633 (WGA-633, Thermofisher Scientific) for 15 min. All bacteria,  
152 except *P. fluorescens* BBc6, *B. ginsengisoli* E3BF7\_7 and *Dyella* sp. E3BF9\_7 that constitutively  
153 expressed GFP, were stained with 0.3 µM 4',6-Diamidino-2-Phenylindole (DAPI, Thermofisher

154 Scientific) for 15 min. Several dyes were used to visualize matrix components: 1X SYPRO Ruby (15 min  
155 incubation; Thermofisher Scientific), DAPI, prodidium iodide ( $1\mu\text{g}\cdot\text{ml}^{-1}$ , 15 mins; Thermofisher Scientific)  
156 or TO-PRO-3 ( $1\mu\text{M}$ , 15 mins; Thermofisher Scientific) were used to stain proteins and extracellular  
157 DNA (eDNA), respectively (Suppl. Table 1). Confocal imaging was performed with a LSM780 Axio  
158 Observer Z1 laser scanning confocal microscope (LSCM, CarlZeiss), equipped with 405, 488 and 633  
159 nm excitation lasers and T-PMT and GaAsp PMT detectors, coupled to ZEN 2.1 lite black software  
160 (CarlZeiss). For all experiments, images were captured with 10x 0.3 NA objective to obtain a complete  
161 view of one fourth of the fungal using a combination of tile scan and Z stack functions (5x5 fields over  
162 the entire depth of the fungal colony). Then images were taken with a 40x 1.2 NA objective to obtain  
163 high resolution zoom images of representative events within the imaged captured at 10x. Data  
164 visualization was performed by 2D maximum intensity projection, using the “Z project” function from Fiji  
165 free software ((Schindelin *et al.*, 2012), <http://fiji.sc/Fiji>).

166 For electron microscopy imaging, fungal colonies or glass fibers were first rinsed with NaCl ( $17\text{ g}\cdot\text{l}^{-1}$ ) and  
167 with sterile water to avoid crystal formation during dehydration step. Then, samples were dehydrated by  
168 freeze-drying and coated with 2 nm of platinum (quartz measurement) under argon plasma ( $2.5\cdot 10^{-2}$   
169 mbar, 35 mA) with a High Vacuum Coater Leica EM ACE600 (Leica). Coated samples were imaged first  
170 with a scanning electron microscope (SEM) equipped with a Field Emission Gun (SIGMA-HPSEM-FEG,  
171 Zeiss) using high resolution “in lens” detector at 1 kV of accelerating voltage. In a second step, some  
172 samples (biofilms grown on glass fibers) were placed in a second SEM (LEO 1450VP W-SEM, Zeiss) to  
173 perform EDS micro-analysis and mappings of elements (20 kV of accelerating voltage at 1 nA of sample  
174 current; Oxford-Instruments INCA MAPS software)

175

#### 176 *Biofilm formation in the presence of Populus roots and ectomycorrhizae*

177 Micro-propagated hybrid poplar (*Populus tremula* × *Populus alba*; INRA clone 717-1-B4) were used to  
178 form mycorrhiza with *L. bicolor* S238N following the method of “*in vitro* sandwich co-culture system”  
179 developed by Felten *et al.* (2009). A mycelium-covered cellophane membrane was placed fungus side  
180 down on the roots of 3 weeks old *Populus* seedlings. Petri dishes were closed with Band-Aids (ensuring  
181 high gas permeability). Cultures were arranged vertically, and the lower part of the dish was covered  
182 with a small black plastic bag to prevent light from reaching the fungus and roots. The co-cultures were  
183 incubated for one month at 24°C and under 16hrs-,photoperiod then the development of mature  
184 ectomycorrhizae was controlled under a stereoscope (CarlZeiss). At this stage, bacterial suspensions  
185 were prepared as described above. Seedlings of *Populus* colonized by *L. bicolor* were transferred in a  
186 large Petri dish filled with sterile PPB (Suppl. Fig. 1). The cellophane membranes were gently detached  
187 by agitation (1 min) and removed. Mycorrhizal seedlings were transferred into a double Petri dish setting

188 containing the bacterial suspension (Suppl. Fig. 1). The double Petri dish was designed to prevent  
189 contact between plant shoot and bacterial suspension. The systems were then incubated at 20°C with  
190 gentle agitation (60 rpm) for 16h. Control treatments (*Populus* plants not inoculated with *L. bicolor*) were  
191 treated similarly. After 16h of incubation, mycorrhizal and non-mycorrhizal seedlings were transferred in  
192 a new double Petri dish to be washed with NaCl and PPB as described above. Each sample was then  
193 examined by confocal microscopy. To obtain transversal cross sections of ectomycorrhizae,  
194 ectomycorrhizae were included in 4% agarose and sectioned with a vibratome (Leica VT1200S) at a  
195 thickness of 30 µm. For each treatment, two independent assays in duplicate were performed.

196

#### 197 *Enzymatic treatment of biofilms*

198 To investigate the role of extracellular DNA (eDNA) in biofilms formation, DNase I (30 Kunitz units.ml<sup>-1</sup>  
199 Qiagen) was added to *P. fluorescens* BBc6 suspensions before the incubation with glass fibers. To  
200 analyze the composition of *P. fluorescens* BBc6 biofilm filaments, biofilms grown for 16 hrs on glass  
201 fibers were treated with proteinase K (60 mAnson units.ml<sup>-1</sup> for 1 hr, ThermoFisher), DNase I (30 Kunitz  
202 units.ml<sup>-1</sup> Qiagen), RNase A (100 Kunitz units.ml<sup>-1</sup>, ThermoFisher) or cellulase R10 (1 mg.ml<sup>-1</sup>, from  
203 *Trichoderma viride*, SERVA). All the enzymatic treatments were performed at room temperature for  
204 2hrs. Samples were observed with laser scanning confocal microscopy (LSCM). Positive controls  
205 were treated similarly. Proteinase K activity was verified with non-fat dried skimmed milk powder as  
206 described by Nygren *et al.* (2007). Cellulase activity was verified with AZCL-HE-Cellulose per the  
207 manufacturer's instructions (0.2 % w/v, Megazyme). For RNase activity, total RNA from human  
208 placenta (1 µg.µl<sup>-1</sup>, Clontech) was treated and degradation was verified by electrophoretic migration  
209 of treated and non-treated RNA (negative control) in 1 % -agarose gel stained with ethidium  
210 bromide.

211

212

## 213 **Results**

214

### 215 *Biofilm formation on L. bicolor S238N hyphae is widespread among soil bacteria.*

216 We previously reported that the MHB *P. fluorescens* BBc6 forms biofilm structures on the  
217 hyphae of *L. bicolor* S238N in *in vitro* setting (Miquel Guennoc *et al.*, 2016). Using the same setting, we  
218 further characterized the process by Laser Scanning Confocal Microscopy (LSCM) and Scanning  
219 Electron Microscopy (SEM). The attachment of individual bacterial cells to the surface of hyphae was  
220 observed after a few minutes of contact between the microorganisms (Fig. 1a, d), followed by the  
221 formation of colonies (after few hours) made of a matrix of several layers of cells engulfing the hyphae

222 (Fig. 1b, e). The colonies kept building up to form mature biofilms after about 20 hours (Fig. 1c, f).  
223 Bacterial cells were encased in a dense matrix of extracellular polymeric substances (Fig. 2a) made of  
224 extracellular DNA (eDNA) and proteins (Fig. 2b). Filaments that bound DAPI stain connected bacterial  
225 cells to each other and anchored the biofilms to the surfaces of the hyphae (Fig. 2 a, c). The substitution  
226 of *L. bicolor* hyphae by glass fibers (similar diameter) in controls also led to biofilm formation around the  
227 glass fibers by *P. fluorescens* BBc6 (Fig. 3a), potentially suggesting that fungal hyphae may be nothing  
228 more than a physical support used by bacteria to establish biofilms. To further test this hypothesis,  
229 biofilm formation on alive and dead fungal colonies was compared. *P. fluorescens* BBc6 formed biofilms  
230 on both living and dead hyphae but the distribution of the biofilms greatly differed between the two  
231 treatments. Although sparse attachment was detected all over the fungal living colonies, mature biofilms  
232 only developed at the actively growing margin of the fungal colony (Fig. 3b). In contrast, biofilms were  
233 found all over dead fungal colonies (Fig 3c) suggesting that specific interactions between bacterial and  
234 fungal cells occur during the formation of biofilms and that fungal hyphae are more than physical  
235 supports.

236 To assess the degree of specificity of the physical interaction between the MHB and the ECM  
237 fungus, thirteen additional cultivable bacteria spanning over a wide range of taxa highly represented in  
238 soil or associated with plants, and with various ecological traits (e.g. MHB, biocontrol, pathogen, Table  
239 1) were tested for their ability to form a biofilm on *L. bicolor* S238N hyphae. All bacteria formed biofilms  
240 around the hyphae at the edge of *L. bicolor* colonies (Suppl. Fig 2).

241

242 *Skeletons of bacterial biofilms are made of DNA filaments.*

243 Biofilms formed by the 14 bacterial strains on hyphae of *L. bicolor* S238N and on glass fibers  
244 were all characterized by the presence of complex networks of filaments stained by DAPI (Suppl. Fig 2).  
245 These filaments could reach a length of several hundred micrometers, and were produced, at least, by  
246 the bacteria since they were also retrieved in BBc6 biofilms formed on glass fibers (Fig. 4a). The  
247 filaments could only be visualized when stained with DNA specific dyes (DAPI, propidium iodide and  
248 TO-PRO-3, Suppl. Table 1A). None of the other dyes tested (e.g. cellulose specific dyes) gave a  
249 positive result (Suppl. Table 1A). In accordance to a DNA composition of the filaments, phosphorous  
250 and nitrogen, two major components of DNA, were both detected at the surface of the filaments by  
251 EDS-SEM elemental mapping (Suppl. Fig. 3). Lastly, a DNase treatment dismantled the biofilms and  
252 disrupted the filaments (Fig. 4b, c) while proteinase K, RNase and cellulase had no visible effect (Suppl.  
253 Table 1B). The filaments were also detected when 2% glucose was added to the incubation medium  
254 (data not shown), indicating that the DNA filaments were not produced as a substitution strategy for  
255 cellulose caused by the absence of carbon sources (Serra *et al.*, 2013).

256

257 *Bacterial biofilm formation on hyphae is restricted to some fungi.*

258 We next tested how widespread is the formation of bacterial biofilm on the hyphae of ten ECM  
259 and non-ECM soil fungi using *P. fluorescens* BBc6 as a model bacterial strain (Table 1). The bacterial  
260 strain formed biofilms on the hyphae of all ECM strains (Table 2, Suppl. Fig. 4) except the Ascomycete  
261 *Tuber melanosporum* (Fig. 5a). Conversely, the bacterial strain produced biofilms on the surface of the  
262 Basidiomycete wood decay *Phanerochaete chrysosporium* but not on the hyphae of the Ascomycete  
263 saprophytes *Aspergillus ustus* AU01 and *Penicillium funiculosum* PF01. In these latter cases *P.*  
264 *fluorescens* BBc6 only attached to the hyphae and never built multilayer biofilms (Fig. 5b). This  
265 contrasts with *Tuber* hyphae on which no attachment at all was visible (Fig 5a).

266

267 *The presence of tree roots and ectomycorrhizae modifies P. fluorescens BBc6 behavior.*

268 Roots and ectomycorrhizae are nutrient hotspots that chemoattract complex communities of  
269 bacteria and that can provide to bacteria alternative habitats from the hyphosphere (Danhorn and  
270 Fuqua, 2007; Bonfante and Anca, 2009). We tested whether the presence of these organs would modify  
271 the behavior of *P. fluorescens* BBc6 using Poplar as a tree model organism. Poplar seedlings were  
272 grown *in vitro* and used to produce ectomycorrhizae with *L. bicolor* S238N. The root system, together  
273 with the associated mycelium, were then incubated with *P. fluorescens* BBc6 bacteria in a liquid setup  
274 without nutrients for 16 hours to assess biofilm formation on free roots, ectomycorrhizae, short- and  
275 long-distance extramatrical mycelium (Suppl. Fig. 1). Bacteria heavily colonized the surfaces of free  
276 roots (Fig 6a), ectomycorrhizae (Fig. 6b), and short-distance extramatrical mycelium (i.e. emerging from  
277 the ECM; Fig. 6c). By contrast, no biofilm was detected on distant hyphae (Fig 6d). To test whether this  
278 absence of biofilm on distant hyphae was caused by a difference of physiology between short- and  
279 long-distance extramatrical hyphae, part of the long-distance extramatrical mycelium was sampled and  
280 transferred to a new plate containing a bacterial inoculum. After 16 hours, a biofilm had formed on the  
281 surface of this “free” long-distance extramatrical mycelium (Fig. 6e), suggesting that the presence of  
282 ectomycorrhizae but not a change in the physiological state of the fungus was responsible for the  
283 change of behavior of the bacterium.

284

## 285 **Discussion & Perspectives**

286

287 In various natural environments, bacteria preferentially live in biofilms that are built on abiotic  
288 surfaces but also on living tissues such as roots (Flemming *et al.*, 2016; Burmølle *et al.*, 2012).  
289 Filamentous fungi represent up to 75% of the subsurface microbial biomass with extended networks of

290 10<sup>2</sup> to 10<sup>4</sup> m length per g of topsoil (Ritz and Young, 2004) and thus could provide immense surfaces for  
291 bacteria to form biofilms. Yet, little is known on the extent on such phenomenon and the consequences  
292 for the fitness of both microorganisms. Our results confirm that life as a biofilm is imprinted in the  
293 genomes of a large taxonomic range of soil bacteria with various life styles and that they are likely to  
294 use the surface of hyphae of fungi such as *L. bicolor* to build biofilms. Our data indicate that such biofilm  
295 formation is likely to occur on both living and dead hyphae (Fig 3). This contrasts with previous reports  
296 on the behavior of *Salmonella enterica* and *Bacillus subtilis* that only formed biofilms on living hyphae of  
297 *Aspergillus niger* (Balbontin *et al.*, 2014; Benoit *et al.*, 2015). However, the use of heat treatment to kill  
298 *A. niger* may have caused a bias by denaturing the structure of the fungal cell wall and thus preventing  
299 the molecular interaction between bacterial cellulose and fungal chitin (Balbontin *et al.*, 2014). In the  
300 present study, bacteria preferentially formed biofilms at the edge of the actively growing colonies while  
301 they colonized the entire fungal colonies when those were killed by fixation in paraformaldehyde (Fig 3)  
302 or ethanol (data not shown). Formation of biofilms on the growing tips of hyphae of fungi by the  
303 bacterium *Burkholderia terrae* was also previously reported (Nazir *et al.*, 2014). Such differential  
304 distribution could be caused by a polarized heterogeneity of the fungal colony, either in the composition  
305 of the fungal cell wall (Latgé, 2007) or in the secretion of nutrients (Webster and Weber, 2007). The  
306 localization of bacterial biofilms on fungal hyphae was also strongly influenced by external biotic cues  
307 such as the presence of roots or ectomycorrhizae (Fig 6). Both plant roots and fungal hyphae produce  
308 various exudates that chemoattract bacteria and can be used as a nutrient source (Johansson *et al.*,  
309 2004; Nazir *et al.*, 2010; Stopnisek *et al.*, 2016; Deveau *et al.*, 2010) but bacteria differ in their abilities  
310 to use these nutrients (Frey *et al.*, 1997). As suggest our results, in the absence of competition between  
311 bacteria, roots and ectomycorrhizae may support more bacterial biofilm formation than the fungus  
312 alone. However, competition between bacteria in natural environment is likely to hinder such behavior  
313 (Förster *et al.*, 2016; Kastman *et al.*, 2016) and it will be necessary to integrate the multiple interactions  
314 that occur within multispecies mixed biofilms in further studies. In addition, settings mimicking the  
315 physico-chemical properties of soils and their texture will allow to shed light onto the parameters that  
316 drive bacterial-fungal interactions in soils.

317

318 If there are evident advantages for bacteria to form biofilms (Balbontin *et al.*, 2014; Stopnisek *et*  
319 *al.*, 2016), the consequences of such biofilm formation for the fungi are not clear. On one hand, bacterial  
320 biofilms could protect the fungal hyphae against grazing, toxic compounds and buffer environmental  
321 variations (Frey-Klett *et al.*, 2011; Kuramitsu *et al.*, 2007). On the other hand, the presence of biofilms  
322 on the growing active area of fungal colonies may limit the capacity of fungi to degrade organic matter  
323 and induce a competition for nutrients between the bacterial community and the hyphae. In this second

324 hypothesis, we expect that fungi, and particularly saprotrophic fungi, would have developed strategies to  
325 block the formation of biofilms on their hyphae (Stöckli *et al.*, 2016; Essig *et al.*, 2014). Our data support  
326 the hypothesis of de Carvalho and colleagues (de Carvalho *et al.*, 2015), in which the mycelium of ECM  
327 fungi is rather permissive to bacterial biofilm formation although exceptions like *T. melanosporum* exist.  
328 Conversely, the inhibition of biofilm formation was more frequent among Ascomycota than  
329 Basidiomycota. Whether this result reflects a reality or is biased by the existence of species-specific  
330 interactions such as demonstrated for AM fungi will need to be further investigated (Toljander *et al.*,  
331 2006; Scheublin *et al.*, 2010). The mechanisms behind the inhibition of biofilm formation by certain fungi  
332 remain also to be discovered. However, our data suggest that they rely on different processes  
333 depending on the fungal species. For instance, preliminary data in *T. melanosporum* suggest that the  
334 fungus would actively inhibit biofilm formation because biofilm formation was observed on dead hyphae  
335 (data not shown).

336

337 The large taxonomic distribution of bacteria able to form a biofilm on *L. bicolor* S238N hyphae  
338 suggest that there was a low degree of specificity of the bacteria towards the fungal host, and thus that  
339 the interaction potentially relied on a common mechanism. Surprisingly, we observed that all bacterial  
340 biofilms were structured by a network of filaments that seem to maintain cell together and to anchor  
341 biofilms to the surface of hyphae and of glass fibers (Fig 2, 4). These skeleton-like structures were  
342 highly reminiscent of the DNA extracellular traps produced by human neutrophils, plant roots and  
343 amoebae to capture bacteria (de Buhr *et al.*, 2016; Zhang *et al.*, 2016; Tran *et al.*, 2016). Our data also  
344 strongly suggest that these skeletons would be made of eDNA. While the presence of eDNA in bacterial  
345 biofilms is now well demonstrated (Flemming and Wingender, 2010), it is often seen as an  
346 anamorphous material. Yet, such eDNA organization into filaments in bacterial biofilms have been  
347 sparsely reported (Böckelmann *et al.*, 2006; Rose *et al.*, 2015; Barnes *et al.*, 2012; Tang *et al.*, 2013;  
348 Gloag *et al.*, 2013; Jurcisek and Bakaletz, 2007; Novotny *et al.*, 2013; Tran *et al.*, 2016; Liao *et al.*,  
349 2014). Together with our data, this suggests that eDNA based skeletons are a common feature of  
350 bacterial biofilms shared between Actinobacteria, Bacteroidetes, Firmicutes,  $\alpha$ -,  $\beta$ - and  $\gamma$ -  
351 Proteobacteria.

352 The role of such eDNA filaments in the formation and functioning of bacterial biofilms remains  
353 elusive. Consistently with the hypothesis that eDNA would serve as a cohesive molecule that maintain  
354 bacterial cells together and anchor them to a surface (Das *et al.*, 2010; Tang *et al.*, 2013), the addition  
355 of DNase at the inoculation time of the bacteria fully blocked the formation of biofilm (data not shown).  
356 Although eDNA skeletons may have additional functions in bacterial biofilms (Gloag *et al.*, 2013;  
357 Doroshenko *et al.*, 2014), it is noteworthy that a broad range of organisms belonging to the Animal,

358 Plant, Protist and Eubacteria Kingdoms all uses DNA for additional purposes than coding genetic  
359 information. Thus DNA, thanks to its adhesive physico-chemical properties, may have an additional  
360 universal function that has been overlooked so far.

361

362 Overall, our work indicates that soil fungi, and most particularly ECM fungi, may often serve as  
363 a support to biofilm formation for a wide range of soil bacteria, in addition to be a source of nutrients and  
364 a potential vector for bacterial mobility. Such biofilm formation on fungal hyphae is likely to be  
365 modulated by numerous biotic factors including roots exudates and fungal activities. Besides the  
366 importance of eDNA based filaments for the building of these biofilms, the molecular dialog involved in  
367 the formation of biofilms on fungal hyphae and the nature of the interaction engaged between the  
368 microorganisms will need to be further investigated.

369

370

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380

381

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512

513 **Tables & Figure legends.**

514

515 **Table 1.** List and characteristics of the microbial strains.

Microbial strains	Taxonomic classification	Gram type	Ecological trait	References
<b>Bacterial strains</b>				
<i>Pseudomonas fluorescens</i> BBc6	Gamma-proteobacteria, Pseudomonadaceae	-	Mycorrhiza helper bacterium	Duponnois & Garbaye 1991
<i>Pseudomonas protegens</i> Pf5	Gamma-proteobacteria, Pseudomonadaceae	-	Biocontrol	Howell & Stipanovic 1979
<i>Pseudomonas fluorescens</i> SBW25	Gamma-proteobacteria, Pseudomonadaceae	-	Plant Growth Promoting & biocontrol	Bailey <i>et al</i> 1995
<i>Pseudomonas fluorescens</i> Pf29A	Gamma-proteobacteria, Pseudomonadaceae	-	Biocontrol	Chapon <i>et al</i> 2002
<i>Pseudomonas sp.</i> GM18	Gamma-proteobacteria, Pseudomonadaceae	-	Mycorrhiza helper bacterium	Labbé <i>et al</i> 2014
<i>Pseudomonas syringae</i> pv. <i>tomato</i> DC3000	Gamma-proteobacteria, Pseudomonadaceae	-	Plant pathogen	Cuppels 1986
<i>Dyella sp.</i> E3BF9_7	Gamma-proteobacteria, Rhodobacteraceae	-	White rot associated bacterium	Hervé <i>et al</i> 2016
<i>Collimonas fungivorans</i> Ter331	Beta-proteobacteria, Oxalobacteraceae	-	Mycophagous bacterium	de Boer <i>et al</i> 2004
<i>Burkholderia ginsengisoli</i> E3BF7_7	Beta-proteobacteria, Burkholderiaceae	-	White rot associated bacterium	Hervé <i>et al</i> 2016
<i>Sinorhizobium meliloti</i> 1021	Alpha-proteobacteria, Rhizobiaceae	-	Nitrogen fixing plant symbiont	Maede <i>et al</i> 1982
<i>Pedobacter sp.</i> D3AIN17A	Bacteroidetes, Sphingobacteriaceae	-	Black truffle associated bacterium	Deveau <i>et al</i> unpublished
<i>Paenibacillus sp.</i> F2001-L	Firmicutes, Paenibacillaceae	+	Endohyphal strain of <i>L. bicolor</i>	Bertaux <i>et al</i> 2003
<i>Bacillus subtilis</i> MB3	Firmicutes, Bacillaceae	+	Mycorrhiza helper bacterium	Duponnois & Garbaye 1991
<i>Bacillus sp.</i> EJP109	Firmicutes, Bacillaceae	+	Mycorrhiza helper bacterium	Poole <i>et al</i> 2001
<b>Fungal strains</b>				
<i>Laccaria bicolor</i> S238N	Basidiomycota, Tricholomataceae	n/a	ECM	Martin <i>et al</i> 2008
<i>Lactarius quietus</i>	Basidiomycota, Russulaceae	n/a	ECM	INRA Nancy
<i>Hebeloma cylindrosporum</i>	Basidiomycota, Cortinariaceae	n/a	ECM	Kohler <i>et al</i> 2015
<i>Piloderma croceum</i>	Basidiomycota, Atheliaceae	n/a	ECM	Kohler <i>et al</i> 2015
<i>Phanerochaete chrysosporium</i> RP78	Basidiomycota, Phanerochaeteaceae	n/a	saprotroph (white rot)	Hervé <i>et al</i> 2016
<i>Thelephora terrestris</i>	Basidiomycota, Thelephoraceae	n/a	ECM	INRA Nancy
<i>Tuber melanosporum</i> Mel28	Ascomycota, Tuberaceae	n/a	ECM	Martin <i>et al</i> 2010
<i>Elaphomyces granulatus</i> PEP	Ascomycota, Elaphomycetaceae	n/a	ECM	Quandt <i>et al</i> 2015
<i>Penicillium funiculosum</i> PF01	Ascomycota, Aspergillaceae	n/a	Saprotroph & plant pathogen	INRA Nancy

<i>Aspergillus ustus</i> AU01	Ascomycota, Aspergillaceae	n/a	Saprotroph	INRA Nancy
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516

517 **Table 2.** Taxonomy and trophic status of fungi tested for bacterial (*P. fluorescens* BBc6) biofilm

518 formation.

Trophic status & taxonomy	Total of strains tested	Biofilm positive	Biofilm negative
ECM	7	6	1
Ascomycota	2	1	1
Basidiomycota	5	5	0
Saprotroph	3	1	2
Ascomycota	2	0	2
Basidiomycota	1	1	0
Taxonomy			
Ascomycota	4	1	3
Basidiomycota	6	6	0

519

520

## 521 Figures

522

523 **Figure 1.** Time course of *P. fluorescens* BBc6 biofilm formation on the surface of *L. bicolor* S238N  
524 hyphae. Confocal microscopy images showing the spatial localization of BBc6 biofilms (green) on *L.*  
525 *bicolor* S238N hyphae (red) and their development over time from early stage attachment (30 min ; a,d)  
526 to colony formation (6h ; b,e) and mature biofilms (20h ; c,f). The yellow arrow points toward the  
527 external edge of the fungal colony. Bottom panels are zoom in of the areas highlighted by white  
528 rectangles in top panels. Fungal hyphae were stained with Wheat Germ Agglutin-AlexaFluor 633 and  
529 bacterial cells were GFP-tagged. Images were obtained via 2D maximum intensity projection of 3D  
530 confocal microscopy images ( $z = 40.5\mu\text{m}$  (a),  $30\mu\text{m}$  (b),  $43.5\mu\text{m}$  (c)). Magnification 40x.

531

532 **Figure 2.** Characterization of the matrix components of *P. fluorescens* BBc6 biofilms on the surface of  
533 *L. bicolor* S238N. **A.** Scanning electron microscopy image of 24h old biofilm showing bacterial cells (\*)  
534 and fungal hyphae (f) encased in a matrix made of aggregates and filaments. **B.** Confocal microscopy  
535 image showing the presence of proteins aggregates (white) and eDNA (blue) in the matrix of 16h old *P.*  
536 *fluorescens* BBc6 (green) biofilm on *L. bicolor* S238N hyphae (red). Proteins and eDNA were stained  
537 with SYPRO Ruby and DAPI, respectively. Magnification 40x. **C.** Confocal microscopy image showing  
538 the presence of filaments stained by DAPI (yellow arrows) connecting hyphae to bacterial cells.  
539 Magnification 40x.

540

541 **Figure 3.** Distribution of *P. fluorescens* BBc6 biofilms on abiotic and biotic surfaces. **A.** Confocal  
542 microscopy image showing *P. fluorescens* BBc6 biofilm formed over glass fibers after 22 hours.  
543 Magnification 40x. **B, C.** Confocal microscopy images showing differential distribution *P. fluorescens*  
544 BBc6 biofilms formed over alive (B) and dead hyphae (C) of *L. bicolor* S238N. *L. bicolor* S238N hyphae  
545 were killed by immersing fungal colonies in 3% paraformaldehyde for 1h followed by 3 repeated washes  
546 in phosphate buffer before inoculating bacteria. Images were obtained via 2D maximum intensity  
547 projection of 3D mosaic confocal microscopy images ( $z = 69\mu\text{m}$  (b),  $32\mu\text{m}$  (c)). Magnification 10x.

548

549 **Figure 4.** Production of eDNA filaments by *P. fluorescens* BBc6 during biofilm formation. **A.** Confocal  
550 images showing mm long filament structures stained by DAPI DNA marker in biofilms of *P. fluorescens*  
551 built on glass fibers. Magnification 40x. **B, C.** Confocal images of TO-PRO-3 stained filaments in *P.*  
552 *fluorescens* BBc6 16 hrs old biofilm on glass fibers before (B) and after DNase treatment (C). White  
553 arrows point at filament positions. Magnification 10 x.

554

555 **Figure 5.** *P. fluorescens* BBc6 does not form biofilm on the hyphae of the Ascomycetes *Tuber*  
556 *melanosporum* (A) and *Aspergillus ustus* (B) after 16hrs of interaction. *T. melanosporum* hyphae were  
557 stained with Wheat Germ Agglutin-AlexaFluor 633 (red). Due to poor staining of *A. ustus* hyphae by  
558 Wheat Germ Agglutin-AlexaFluor 633, *A. ustus* hyphae were imaged using transmitted light.  
559 Magnification 40x.

560

561 **Figure 6.** Differential distribution of *P. fluorescens* BBc6 biofilms on Poplar roots, ectomycorrhizae and  
562 extramatrical mycelium after 16 hrs of interaction. **A.** Confocal image showing *P. fluorescens* BBc6  
563 (green) colonization of Poplar roots (blue). **B.** Transversal section of *L. bicolor* S238N (red)– Poplar  
564 (blue) ectomycorrhizae colonized by *P. fluorescens* BBc6 (green). **C.** Confocal image of *L. bicolor*  
565 S238N extramatrical hyphae (blue) surrounding Poplar root (red) and colonized by *P. fluorescens* BBc6  
566 (green). **D.** Confocal image showing *L. bicolor* S238N extramatrical hyphae (red) distant from root and  
567 ectomycorrhizae that are not colonized by *P. fluorescens* BBc6 (green) in the presence of Poplar root  
568 system. **E.** Confocal image showing *L. bicolor* S238N extramatrical hyphae (red) distant from root and  
569 ectomycorrhizae colonized by *P. fluorescens* BBc6 (green) in the absence of Poplar root system.  
570 Magnification 40x.

571 *L. bicolor* S238N hyphae were stained with Wheat Germ Agglutinin-AlexaFluor 633 (red), Poplar root cells  
572 and eDNA were visualized with a combination of DAPI staining and autofluorescence (blue) and  
573 bacterial cells were GFP-tagged (green). All images were obtained via 2D maximum intensity projection  
574 of 3D confocal microscopy images ( $z = 60 \mu\text{m}$  (a),  $57 \mu\text{m}$  (b),  $68 \mu\text{m}$  (c),  $46 \mu\text{m}$  (d),  $29 \mu\text{m}$  (e)).  
575 Magnification 40x.

576 mr: main root, rh: root hairs, exm :extramatrical mycelium.

577  
578

579 **Supplemental Fig. 1.** Experimental setup used to analyze biofilm formation during tripartite interaction  
580 between *P. fluorescens* BBc6, *L. bicolor* S238N and *Populus tremula x alba*. Mycorrhizal seedlings were  
581 first produced using the *in vitro* sandwich co-culture system (Felten et al. 2009). One-month-old  
582 mycorrhizal seedlings were then used to analyze *in vitro* biofilm formation on ectomycorrhizae, non-  
583 mycorrhized roots, and extramatrical mycelium.

584

585 **Supplemental Fig. 2.** Biofilm formation of the hyphae of *L. bicolor* S238N by bacteria as visualized by  
586 confocal microscopy. Fungal hyphae were stained with Wheat Germ Agglutinin-AlexaFluor 633 (red) and  
587 bacteria with DAPI (blue), except *P. fluorescens* BBc6, *B. ginsengisoli* E3BF7\_7 and *Dyella* sp.  
588 E3BF9\_7 that constitutively expressed GFP (green). eDNA filaments are highlighted by yellow arrows in  
589 caption boxes. Images were obtained via 2D maximum intensity projection of 3D confocal microscopy  
590 images Magnification 40x.

591

592 **Supplemental Fig. 3.** Elemental mapping of P (b, c), N (d, e) and Si (f, g) of BBc6 filaments and biofilm  
593 matrix on glass fibers obtained by EDS Spectrometry coupled to SEM imagery. Electron image: a, raw  
594 elemental map; b, d and f, overlay of electron image and elemental map : c, e, g

595

596 **Supplemental Fig. 4.** Biofilm formation by *P. fluorescens* BBc6-GFP on hyphae of soil fungi. Fungal  
597 hyphae were stained with Wheat Germ Agglutinin-AlexaFluor 633 (red), bacterial cells were GFP-tagged  
598 and eDNA was stained with DAPI (blue). Due to poor staining of *T. terrestris*, *A. ustus* and *P.*  
599 *funiculosum* hyphae by Wheat Germ Agglutinin-AlexaFluor 633, hyphae of these fungi were imaged using  
600 transmitted light, coloured in red. Images were obtained via 2D maximum intensity projection of 3D  
601 confocal microscopy images. Magnification 40x.

602











