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2

3 **Title:** Visualization of root extracellular traps in an ectomycorrhizal woody plant (*Pinus*

4 *densiflora*) and their interactions with root-associated bacteria

5

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17 **Main Conclusion:** Extracellular traps in the primary root of *Pinus densiflora* contribute to root-

18 associated bacterial colonization. Trapped rhizobacteria induce the production of reactive oxygen

19 species in root-associated, cap-derived cells.

20

21 **Abstract**

22 Ectomycorrhizal (ECM) woody plants, such as members of Pinaceae and Fagaceae, can acquire

23 resistance to biotic and abiotic stresses through the formation of mycorrhiza with ECM fungi.

24 However, germinated tree seedlings do not have mycorrhizae and it takes several weeks for

25 ectomycorrhizae to form on their root tips. Therefore, to confer protection during the early

26 growth stage, bare primary roots require defense mechanisms other than mycorrhization. Here,

27 we attempted to visualize root extracellular traps (RETs), an innate root defense mechanism, in

28 the primary root of *Pinus densiflora* and investigate the interactions with root-associated bacteria

29 isolated from ECM and fine non-mycorrhizal roots. Histological and histochemical imaging and

30 colony forming unit assays demonstrated that RETs in *P. densiflora*, mainly consisting of root-

31 associated, cap-derived cells (AC-DCs) and large amounts of root mucilage, promoted bacterial

32 colonization in the rhizosphere, despite also having bactericidal activity via extracellular DNA.

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33 Four rhizobacterial strains induced the production of reactive oxygen species (ROS) from host

34 tree AC-DCs without being excluded from the rhizosphere of *P. densiflora*. In particular,

35 applying two *Paraburkholderia* strains, PM O-EM8 and PF T-NM22, showed significant

36 differences in the ROS levels from the control group. These results reveal an indirect

37 contribution of rhizobacteria to host root defense, and suggest that root-associated bacteria could

38 be a component of RETs as a first line of defense against root pathogens in the early growth

39 stage of ECM woody plants.

40

41 **Keywords:**

42 Border cells; Japanese red pine; *Paraburkholderia*; Primary root; Rhizobacteria; Root mucilage

43

44 **Abbreviations**

45 **AC-DCs:** root-associated, cap-derived cells, **CFU:** colony-forming unit, **CV:** crystal violet,

46 **CW:** calcofluor white, **exDNA:** extracellular DNA, **RAM:** root apical meristem, **RBC:** root

47 border cell, **RBLC:** root border-like cell, **RETs:** root extracellular traps, **ROS:** reactive oxygen

48 species

Introduction

Plants have root apical meristems (RAMs) on the root tip, which is a vital organ that controls cellular division and differentiation (Motte et al. 2019). Roots are constantly exposed to various biotic and abiotic stresses during elongation through soil (Cramer et al. 2011; Suzuki et al. 2014; Ganesh et al. 2022). Among stressors, pathogen attack is a major cause of mortality of juvenile plants (Atkinson and Urwin 2012; Gonthier and Nicolotti 2013). Plants repel or mitigate pathogen invasion and infection via innate defense responses, such as pathogen/microbe-associated molecular pattern (PAMP/MAMP)-triggered immunity (Huot et al. 2014; Cook et al. 2015). Furthermore, numerous studies have shown that host roots recruit nonpathogenic, root-associated bacteria by releasing various root exudates (Badri and Vivanco 2009; Sasse et al. 2018). Such bacteria produce exopolysaccharides, antipathogenic metabolites, and phytohormones, which in turn help control the rhizosphere environment (De Vleesschauwer and Höfte 2009; Kuzyakov and Razavi 2019; Mohanram and Kumar 2019). With the development of high-throughput sequencing technologies, we have come to better understand the interactions between host plants and pathogenic, commensal, and mutualistic rhizospheric microorganisms, as well as their impacts on the health and productivity of host plants.

Rhizodeposits include water-soluble and volatile compounds that are released from host roots, and are involved in biological defense. The root cap is made up of root border cells (RBCs) and root border-like cells (RBLCs), which cover vital tissues and release single or connected cells into the soil. Collectively, RBCs and RBLCs are termed root-associated, cap-derived cells (AC-DCs) (Hawes and Lin 1990; Hawes et al. 2003; Driouich et al. 2019). Studies have revealed that these cells remain alive after release from the root cap (Hawse and Pueppke 1986) and secrete mucilage composed of a mixture of polysaccharides, proteoglycans, extracellular DNA (exDNA), defensin peptides, reactive oxygen species (ROS), and various secondary metabolites (Plancot et al. 2013; Wen et al. 2017; Driouich et al. 2019; Driouich et al. 2021). These

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74 structures, consisting of AC-DCs and their secretions that spread to encompass the root cap, are

75 known as root extracellular traps (RETs) because of similarities with neutrophil extracellular

76 traps, which are part of the innate immune system in animals (Brinkmann et al. 2004; Driouich et

77 al. 2013). Studies on RETs have mainly focused on their defense functions against root

78 pathogens. Most studies have used herbaceous plant species, such as *Arabidopsis* (*Arabidopsis*

79 *thaliana* [L.] Heynh), cotton (*Gossypium* spp.), maize (*Zea mays* L.), and pea (*Pisum sativum* L.)

80 (Hawes et al. 2003; Vicré et al. 2005; Wen et al. 2009; Hawes et al. 2016; Fortier et al. 2023). By

81 contrast, the roles of RETs in woody plants remain poorly understood, and only a few cases have

82 been investigated, including in *Acacia mangium* Willd. (Endo et al. 2011), grapevine (*Vitis*

83 *riparia* × *Vitis labrusca*) (Liu et al. 2019), and some species in arid regions (*Balanites*

84 *aegyptiaca* [L.] Del. fruits, *Acacia raddiana* Savi, and *Tamarindus indica* L.) (Carreras et al.

85 2020).

86   Pinaceae is a representative ectomycorrhizal (ECM) tree family that inhabits temperate and

87 boreal forests in the Northern Hemisphere. This family acquires resistance to biotic and abiotic

88 stresses, including pathogen attacks, via belowground ECM associations. Such resistance allows

89 Pinaceae species to invade non-forested sites and survive in the seedling stage (Martín-Pinto et

90 al. 2006; Zhang et al. 2017; Policelli et al. 2019). However, ECM fungi generally associate with

91 only fine root tips (≤ 2 mm in diameter), which generate from lateral roots. This suggests that

92 there is a period between seed germination and ECM root formation of weakened defense

93 responses during root formation. Therefore, we hypothesized that other defense mechanisms

94 confer protection in the early growth stage, and focused our investigations on RETs and root-

95 associated bacteria.

96   We elucidated the features and roles of RETs in the early stage of germination of ECM tree

97 species. To this end, we visualized the RETs of an ECM woody gymnosperm, Japanese red pine

98 (*Pinus densiflora* Sieb. et Zucc.), investigated whether RETs in the host tree species for early

4

99 growth kill root-associated bacteria or promote their colonization, and evaluated the effects of

100 those bacteria on the defense responses of host tree AC-DCs.

101

102 **Materials and Methods**

103 **Plant material**

104   *Pinus densiflora* seeds were immersed in distilled water in darkness at 4℃ overnight (for 12–

105 18 h). They were shaken in a neutral detergent solution for 1 min using a magnetic stirrer and

106 rinsed with tap water. Intact seeds were surface sterilized with 30% (v/v) hydrogen peroxide for

107 30 min and rinsed several times with sterile water; then they were sown on sterile filter paper

108 moistened with sterile water and incubated under aseptic conditions in darkness at 25℃ for 4–14

109 days (mainly those germinated on the fourth or fifth day of culture with a root length < 2 cm

110 were used). Before separation of RETs from the root cap for microscopic observation or

111 experimentation, germinated seeds were soaked on sterile filter paper soaked with sterile water

112 and incubated overnight under the same conditions as described above. Hereafter, this process is

113 referred to as the swelling treatment (Fig. 1a, b).

114

115 **Isolation and identification of rhizobacterial strains**

116   Table 1 lists the four bacterial strains used in this study. To obtain rhizobacteria, we collected

117 root system samples of mature *P. densiflora* trees in August 2020 at Ome Forest, a temperate

118 secondary forest in Ome, Tokyo, Japan (35°47'50.4"N, 139°15'44.4"E), and the University of

119 Tokyo Tanashi Forest, a planted forest in Nishitokyo, Tokyo, Japan (35°44'21"N, 139°32'15"E).

120 The samples were gently washed in tap water with a brush under a stereomicroscope (Leica

121 MZ16; Leica Microsystems, Wetzlar, Germany). Five ECM root tips of the same morphotypes

122 and the same number of non-mycorrhizal (NM) root tips were selected from each sample. They

123 were transferred to a 1.5 mL tube containing 1 mL sterile water and shaken for 1 min using a

5

124 vortex to remove fine particles. After repeating this process three times, each root tip was

125 homogenized using a micropestle and suspended in 1 mL sterile water. The suspension was used

126 as a stock solution, which was serially diluted up to 10,000 times, and 100 µL each dilution was

127 spread on yeast glucose (YG) agar medium containing 1.0 g yeast extract, 1.0 g glucose, 0.3 g

128 K_2HPO_4, 0.3 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 15 g agar, and 1 L distilled water. All medium

129 plates were incubated in darkness at 25°C for 2–7 days, and bacterial colonies generated on the

130 plate were randomly isolated, of which four strains with different colony morphologies were

131 selected for this study.

132   Species identification of the four bacterial strains was based on the 16S ribosomal RNA

133 (rRNA) gene sequence. The V1–V9 regions of the 16S rRNA genes were amplified by direct

134 polymerase chain reaction (PCR) from a single colony using EmeraldAmp PCR Master Mix

135 (Takara Bio, Shiga, Japan), and the universal primer pair 27F and 1492R (Weisburg et al. 1991).

136 The PCR cycling conditions were as follows: denaturing at 94°C for 1 min; 40 cycles of 98°C for

137 10 s, annealing at 55°C for 30 s, and 72°C for 90 s; and a final extension at 72°C for 7 min.

138 Successfully amplified PCR products were purified using illustra ExoProStar (GE Healthcare,

139 Buckinghamshire, UK) and submitted to Macrogen DNA Sequencing Service (Macrogen,

140 Tokyo, Japan) for Sanger sequencing. Four universal primers (27F, 518F, 800R, and 1492R)

141 were used as sequencing primers to obtain nearly complete sequences (>1300 bp). After

142 checking the quality check of the obtained sequences with reference to the original

143 chromatograms, bacterial species were identified at least to the genus level via a BLAST search

144 of the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). These sequences were

145 deposited in the DNA Database of Japan (https://www.ddbj.nig.ac.jp/index-e.html) under the

146 accession numbers LC743733–LC743736.

147

148 **Histochemical staining and microscopy of RETs**

149   Root tips from *P. densiflora* in the early germination stage were mounted on a glass slide for

150   stereomicroscopy and light microscopy. Components of RETs were mounted in sterile water or

151   following staining on a glass slide for bright-field and fluorescence microscopy using a light

152   microscope (Olympus BX50; Olympus, Tokyo, Japan). All staining and observation processes

153   were conducted on at least three technical replicates.

154   To visualize the viability of AC-DCs shed from the root cap, live and dead cells were stained

155   with fluorescein diacetate solution ($1\ \mu g \cdot mL^{-1}$ in phosphate-buffered saline [PBS]) and 0.01%

156   (v/v) Evans blue solution, respectively. Cells were stained and observed within 15 min of

157   separation from the root. The same process was also performed on the separated samples

158   incubated in a 1.5 mL tube containing 200 µL sterile water at 25°C for 2, 4, 7, 14, and 28 days.

159   Five replicates were prepared for each timepoint.

160   AC-DCs were stained using 1% or 3% (v/v) crystal violet (CV) solution, and the mucilage

161   layer was visualized using CV of the same concentration and 0.4% (v/v) India ink solution;

162   sterile water was used as the solvent for both solutions.

163   The CV solution and calcofluor white M2R solution ($1\ mg \cdot mL^{-1}$ in sterile water) were used to

164   clarify the origin of branched strands frequently found as AC-DCs shed. Moreover, actin

165   filaments in AC-DCs were visualized under the following conditions, at room temperature: cells

166   were fixed with 4% (v/v) paraformaldehyde in PBS (pH 7.0) for 15 min, rinsed with PBS for 30

167   s, incubated in 0.1% (v/v) Triton X-100 solution in PBS for 15 min, rinsed with PBS for 30 s

168   twice, stained with 0.1 µM Acti-stain 555 fluorescent phalloidin (Cytoskeleton Inc., Denver, CO,

169   USA) for 30 min, rinsed with PBS for 30 s three times, stained with 4′,6-diamidino-2-

170   phenylindole (DAPI) as a counterstain for 30 s, and rinsed with PBS for 30 s.

171   ExDNA released from AC-DCs was labeled using 1 µM SYTOX Green (Thermo Fisher

172   Scientific, Waltham, MA, USA). Stock solutions were made following the manufacturer's

173   instructions.

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Visualization of rhizobacteria trapped by RETs

For the observations, four bacterial strains, mainly *Bacillus* sp. strain O-EM7 (BC O-EM7)

and *Paraburkholderia* sp. strain O-NM9 (PS O-NM9) were pre-cultivated on YG agar medium

at 25°C for 2 days, and then were suspended in sterile water and adjusted to an optical density at

600 nm (OD_{600}) of 0.05. Next, the tip of a primary root was immersed in a 1.5 mL tube

containing 200 µL suspension and was incubated in darkness at 25°C for 8 h or 2 days. After

incubation, RETs were detached from the root cap using tweezers, rinsed with sterile water

twice, then stained with 3% CV solution and 1 µM SYTOX Green.

Colony-forming unit assay

We performed a colony-forming unit (CFU) assay to confirm the effects of the presence or

absence of RETs on bacterial colonization in the early stage of *P*. *densiflora* rhizosphere

development. Root tips, processed via swelling treatment, were immersed in bacterial

suspensions ($OD_{600} = 0.05$), and RETs were removed under aseptic conditions before and 2 days

after incubation at 25°C, respectively. Root tips without RETs removed were used as the control

(n = 5). Incubated samples were separated from the seed, leaving a root tip of 5 mm, gently

rinsed with sterile water twice, homogenized using a micropestle, and suspended in 1 mL sterile

water. The suspensions were used as a stock solution, serially diluted up to 100,000 times, and

100 µL each dilution was spread on two dishes of YG agar medium per dilution step. After

incubation at 25°C for 2 days, bacterial colonies formed on the medium were counted; the

average number of colonies in two dishes that fell within 30–300 was adopted, and the values

were log-transformed (Log10 CFU) for statistical analysis.

Detection of total ROS in response to contact with rhizobacteria

199   To evaluate the defense response of the RETs induced by contact with rhizobacteria, we

200   examined ROS production, which is an early defense signal in plant root immunity (Boller and

201   Felix 2009). After swelling treatment, RETs were stripped from the root tip, immersed in 200 µL

202   each bacterial suspension (OD_{600} = 0.05), and incubated in darkness at 25°C for 4 h. In addition,

203   RETs were treated with 1 µM flg22 (Alpha Diagnostic International Inc., San Antonio, TX,

204   USA), a representative MAMP peptide derived from bacterial flagella (Millet et al. 2010). Sterile

205   water was used as the control, and five replicates of each treatment were performed. After

206   rinsing twice with Hank's balanced salt solution without phenol red (HBSS-), RETs were stained

207   using the fluorescent probe ROS Assay Kit -Highly Sensitive DCFH-DA- (Dojindo Laboratories,

208   Kumamoto, Japan) at 25°C for 30 min. The working solution was prepared following the

209   manufacturer's instructions. Stained samples were rinsed twice with HBSS-, and then five

210   random locations per sample were imaged under fluorescence microscopy within 2 h of staining

211   using the following conditions (except when images were captured at 1000× magnification):

212   2040 × 1536 pixels; 200× magnification; ISO200; 12 ms exposure; RGB values of 0.7:1.0:2.1; 8

213   bits; and tiff. extension. Total ROS production, here considered to indicate the degree of cellular

214   sensitivity to the treatments, was calculated as the relative fluorescence intensity per sample,

215   using Fiji (ImageJ ver. 1.53q) software (Schindelin et al. 2012). First, the original image was

216   divided into three colors (red, blue, and green), and red was subtracted from green. Then, an

217   image was generated using the Li model and a range of 25–255 as the threshold value. Finally,

218   the number of pixels corresponding to the threshold was counted.

219

220   **Image processing**

221   Fiji, Inkscape ver. 1.0 (https://inkscape.org/), and GIMP ver. 2.10.20 (https://www.gimp.org/)

222   software were used to export and process each figure. Except for those used for the image

223   analyses, when processing images after capturing, the entire image was altered and only

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224 brightness and contrast were modulated. Movies (Online Resources 1–8) were processed using

225 DaVinci Resolve ver. 18.1.2 (https://www.blackmagicdesign.com/products/davinciresolve/)

226 under the same conditions.

227

228 **Statistical analysis**

229   The significance of differences among treatments in the CFU and ROS detection assays were

230 analyzed by one-way analysis of variance using the Tukey's HSD test. Data processing and

231 analysis, and graph plotting were performed using R ver. 4.0.4 (R Core Team 2021).

232

233 **Results**

234 **Visualization of RETs in the primary root of *P. densiflora***

235   Figure 1 presents an overview of the RETs and their major components in the primary root of

236 *P. densiflora*. RBC shedding, RBLC detachment, and root mucilage percolation from the root

237 cap were immediately observed when root tips that had not yet undergone swelling treatment

238 were immersed in the solutions (Fig. 1c, e, and f; Online Resources 1 and 2). The root cap

239 released mainly elliptic and oblong RBCs from the apex, and sheath-shaped and long layers of

240 RBLCs from the lateral sides. CV readily stained each component, revealing their dispersal to

241 cover the root tip; membranous mucilage was visualized particularly well (Fig. 1f, Fig. 2). Most

242 of the AC-DCs remained viable in sterile water immediately after isolation from the root cap

243 (Fig. 1d). We confirmed vigorous cytoplasmic streaming initially and even after 7 days of

244 isolation (Online Resources 3–5); some cells remained viable until day 28 (Fig. S1). However,

245 cells mounted without liquid wilted and died quickly. Branched strands were observed to

246 protrude from both living and dead cells, particularly at the joints between RBLCs and at the

247 longitudinal tip of the RBCs (Fig. 1g–i). Damaged AC-DCs experiencing plasmolysis tended to

248 discharge copious amounts of strands compared to live cells (Fig. 1g). They were readily stained

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249   with CV and CW, and slightly stained with Acti-stain 555 phalloidin; however, no labeling was

250   observed after application of two fluorescent dyes for DNA, DAPI and SYTOX Green (Fig. 1g–

251   l). Actin filaments in the AC-DCs had structures similar to the strands, but we could not confirm

252   whether they were identical (Fig. 1j). SYTOX Green stained exDNA, which visualized their

253   spread in thread-like or web-like structures (Fig. 1m–o, Fig. 3); these structures were observed

254   during unraveling of the spherical structure of the cell nucleus (Fig. 3a, b), and some structures

255   spread more than five times the area of a single RBC (Fig. 3c). The unfolding of exDNA was

256   found both with or without microorganisms, and we could not confirm active secretion during

257   our observations.

258

259   **Trapping of rhizobacteria by RETs**

260   We demonstrated that rhizobacterial cells were trapped by root mucilage and exDNA using

261   CV and SYTOX Green, respectively (Fig. 2, Fig. 3). Bacterial cells, which exhibited active

262   swarming in suspension, showed minimal movement after adhering to the mucilage, and were

263   not liberated when the solution was gently agitated (Fig. 2c, e, and f; Online Resources 6–8).

264   Branched strands, similar to frameworks, were also frequently entangled in the mucilage layer

265   with bacterial cells (Fig. 2d). SYTOX Green is a DNA-specific dye that cannot penetrate living

266   cell membranes (Wen et al. 2017); hence, only dead cells, including bacteria, show a

267   fluorescence response. SYTOX Green staining visualized some dead bacterial cells trapped by

268   exDNA that had spread in thread-like or web-like structures within the RETs (Fig. 3c, d, and e).

269   However, bacteria trapped by exDNA were localized compared to those in the mucilage layer,

270   and many living bacterial cells attached to the mucilage were observed under bright-field

271   conditions (Fig. 3f).

272   The CFU assay results indicated that RETs could contribute to bacterial colonization in the

273   early germination stage of the rhizosphere of *P. densiflora*. By contrast, RET removal (Fig. 4a)

11

274 tended to reduce CFU counts, particularly those removed after incubation, which significantly

275 impaired the CFU counts of all bacterial strains (Fig. 4b). There were no differences in the

276 morphological features of bacterial colonies formed on the medium among these treatments.

277

278 **Production of ROS in response to rhizobacteria**

279 Fluorescent staining for total ROS revealed the production of ROS in AC-DCs (Fig. 5). An

280 early fluorescence response to bacterial MAMP perception, the oxidative burst (Boller and Felix

281 2009; Zipfel 2009), could be roughly divided into two patterns based on the fluorescence signal:

282 in the first pattern, fluorescence was isolated to cellular organelles; in the second pattern,

283 fluorescence was observed in the whole cell, except the nucleus (Fig. 5b). A comparison of the

284 relative fluorescence intensity based on image analysis revealed that co-incubation with

285 rhizobacterial strains tended to enhance the total ROS production of AC-DCs (Fig. 5c). Two

286 *Paraburkholderia* strains, PM O-EM8 and PF T-NM22, significantly differed from the control

287 treatment, and the latter had the highest values. However, BC O-EM7 and PS O-NM9 did not

288 result in significant differences in the fluorescence response compared to the control group;

289 fluorescence responses were also detected in a wide range of intracellular organelles such as

290 plastids, mitochondria, and peroxisomes, similar PM O-EM8 and PF T-NM22 (Fig. 5a, c). In

291 addition, the ROS levels detected after flg22 treatment did not significantly differ from the

292 control, and were lower than those after co-incubation with the bacterial suspensions.

293

294 **Discussion**

295 The number of AC-DCs produced and the pattern they form vary among plant species. For

296 instance, the root cap of *P. sativum* produces many RBCs, whereas *A. thaliana* mainly releases

297 cells connected in layers (Hawes et al. 1998; Vicré et al. 2005; Driouich et al. 2019). Hamamoto

298 et al. (2006) demonstrated that these differences among dicotyledonous angiosperms are

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299   attributable to variation in RAM organization structures. Meanwhile, Carreras et al. (2020)

300   observed that two Fabaceae tree species, *A. raddiana* and *T. indica*, have open RAMs, similar to

301   *P. sativum*, and yet release mainly sheaths of RBLCs. Our observations of the woody

302   gymnosperm *P. densiflora* matched neither *A. thaliana* nor *P. sativum* but were relatively similar

303   to *A. raddiana* among the species studied to date. Therefore, our results partly corroborate those

304   of Hamamoto et al. (2006) in that *P. densiflora* has a different RAM structure than *A. thaliana*

305   and *P. sativum* (Imaichi et al. 2018). These findings suggest that additional factors, such as

306   differences between angiosperms and gymnosperms, differences between dicots and monocots,

307   and the types of symbioses with microorganisms, should be considered in addition to RAM

308   structure. Furthermore, the pectin-degrading enzymes pectin methylesterase and

309   polygalacturonase are reportedly involved in such cell separation (Hawes and Lin 1990; Wen et

310   al. 1999; Driouich et al. 2007). In *A. thaliana*, Kamiya et al. (2016) showed that three NAC

311   transcription factors, *SOMBRERO*, *BEARSKIN* (*BRN*) *1*, and *BRN2*, regulate the expression of

312   *ROOT CAP POLYGALACTURONASE* (*RCPG*) in polygalacturonase secretion; of these, at least

313   *BRN1* directly binds to *RCPG* promoter. Karve et al. (2016) also revealed that the transcription

314   factor NIN-LIKE PROTEIN 7 controls the cell wall-loosening enzyme CELLULASE 5, thereby

315   enabling the release of RBLCs in *A. thaliana*. Comparing the expression dynamics of target

316   genes among plant species may help categorize their cell detachment patterns.

317   In *Pinus* species, the viability and long-term survival of AC-DCs still require examination,

318   although such cells have been assessed immediately after detachment (Hawse and Pueppke

319   1986). Our tests, performed under non-nutritional liquid conditions, support the previous study,

320   which used *Glycine max* and *P. sativum* (until 31 days), suggest that the AC-DCs of *P.*

321   *densiflora* could survive for more extended periods under favorable conditions. In addition,

322   given that RET components are produced from living AC-DCs (Driouich et al. 2019), these

323   findings indicate that soil nutrient deficiencies or drought stress cause dysfunction of RETs

324   under field conditions and may lead to the death of young seedlings.

325   We confirmed that the strand structures were not stained with either of two DNA-specific

326   fluorescent probes. A recent study described entangled strands readily stained with CV as

327   "barbed wire" (Wen et al. 2017, p. 974) structures, and they could be digested by DNase I or II

328   (Wen et al. 2017; Huskey et al. 2019). Moreover, Ropitaux et al. (2019) reported that cellulose

329   and xyloglucan present as a dense fibrous network in the root mucilage and maintain AC-DC

330   attachments. These findings are reasonably consistent with our histochemical observations using

331   CW (staining β-linked polysaccharides) and phalloidin (staining F-actin), indicating that the

332   strands observed in this study differ from exDNA, and their origin is the cell wall or

333   cytoskeleton. Although the role of branched strands in RETs remains unclear, we postulate that

334   they strengthen the structure of mucilage and function as a physical scaffold that binds bacteria,

335   which are found frequently in the mucilage layer. This hypothesis does not conflict with the facts

336   that RBCs can show selectivity for bacteria (Hawes and Pueppke 1989) and root mucilage is a

337   carbon source for rhizobacteria, influencing their community compositions (Knee et al. 2001;

338   Benizri et al. 2007). Thus, these strands may be considered a nonlethal and mucilage-coated

339   component for rhizobacterial trapping, which implies that AC-DCs have some functions even

340   after cell death.

341   Based on our observations, bacterial cells did not survive trapping by exDNA. Defensin

342   peptides and exDNA have bactericidal functions in RETs. In exDNA, histone H4 (the only

343   DNA-binding protein found in plants) induces microorganism death by disrupting their cell

344   membranes (Wen et al. 2007; Hawes et al. 2012; Driouich et al. 2019; Monticolo et al. 2020).

345   Thus, exDNA from AC-DCs in *P. densiflora* likely has a lethal effect against bacteria, whereas

346   while root mucilage enables nonlethal trapping. Our CFU assay-based results supported these

347   observations; consequently, the RETs of *P. densiflora* should facilitate commensal and beneficial

348   bacterial colonization in the rhizosphere despite being capable of killing bacteria. However,

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349　additional studies will be needed to fully elucidate the effects of exDNA against rhizobacteria;

350　for instance, they may have a controlling effect to prevent bacterial overgrowth in the

351　rhizosphere. Future studies also should focus on the immune-evasive and -suppressive

352　capabilities of bacteria, i.e., degrading exDNA, hiding MAMPs, and modulating hormonal

353　signaling pathways (Tran et al. 2016; Yu et al. 2019; Teixeira et al. 2021).

354　　　The four rhizobacterial strains induced ROS production from the host tree AC-DCs of the *P.*

355　*densiflora*. A recent study on *A. thaliana* elucidated a feedback loop, that is, an interaction

356　between host plant root and beneficial bacteria; the study showed that bacterial colonization

357　elicited a root immune response and ROS production, followed by auxin stimulation, thereby

358　promoting bacterial survival in the rhizosphere (Tzipilevich et al. 2021). Our results may be

359　relevant to this cycle in that the bacterial strains were not excluded from the rhizosphere.

360　Moreover, we assessed three *Paraburkholderia* strains, of which PF T-NM22 induced a notably

361　high level of ROS production. The four bacterial strains investigated in this study belong to two

362　genera, *Bacillus* and *Paraburkholderia*. Both genera are common beneficial rhizobacteria

363　(Santoyo et al. 2016), and the latter prime plant immune responses, functioning as a first line of

364　defense against pathogens (Carrión et al. 2018; Tringe 2019; Leitão et al. 2021; del Carmen

365　Orozco-Mosqueda et al. 2022). Therefore, our results show the indirect contribution of

366　rhizobacteria to host root defense and indicate that root-associated bacteria could be a component

367　of RETs, which is equivalent to "an additional layer of the plant immune system" (Teixeira et al.

368　2019, p. 13).

369　　　The present findings reveal that RETs function in the early growth stage of *P. densiflora*. To

370　the best of our knowledge, this is the first study to investigate RETs in an ECM woody

371　gymnosperm and their influence on rhizobacterial colonization. However, our findings are

372　limited to only the primary roots, given that root morphology changes anatomically during

373　growth and turnover (Brunner and Scheidegger 1992; McCrady and Comerford 1998; Peterson et

15

374 al. 1999), and the mechanisms of metabolites vary among root zones within an individual root

375 (Sasse et al. 2018). Thus, it would be beneficial to investigate whether RETs function in mature

376 lateral root systems.

377

378 **Author Contribution Statement**

379 MS conceived the research plans and experimental designs; NM and KF supervised and

380 improved them. MS performed the experiments, microscopic observations, and data analyses and

381 drafted the manuscript; NM and KF critically reviewed and provided feedback. All authors

382 contributed to the revision of the manuscript and approved the final version for submission.

383

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388 **Declarations**

389 **Conflict of interest**

390 The authors have no conflicts of interest associated with the article.

391

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Table 1 Root-associated bacterial strains isolated from the rhizosphere of *Pinus densifora*

Strain	Abbreviation	Isolation source (location)	Accession no.	Top BLAST hit in GenBank (acc. no.)	Similarity (%)
Bacillus sp. strain O-EM7	BC O-EM7	ECM root tips (Ome, Tokyo)	LC743733	*Bacillus cereus* strain CASMBAUDAL1 (KM524118)	99.58
Paraburkholderia sp. strain O-EM8	PM O-EM8	ECM root tips (Ome, Tokyo)	LC743734	*Paraburkholderia metrosideri* strain 17G39-22 (MH934925)	100
Paraburkholderia sp. strain O-NM9	PS O-NM9	NM root tips (Ome, Tokyo)	LC743735	*Paraburkholderia sediminicola* strain HU2-65W (MN727305)	99.18
Paraburkholderia sp. strain T-NM22	PF T-NM22	NM root tips (Tanashi, Tokyo)	LC743736	*Paraburkholderia* sp. JSA6 (LC682224)	99.66

ECM, ectomycorrhizal; NM, non-mycorrhizal
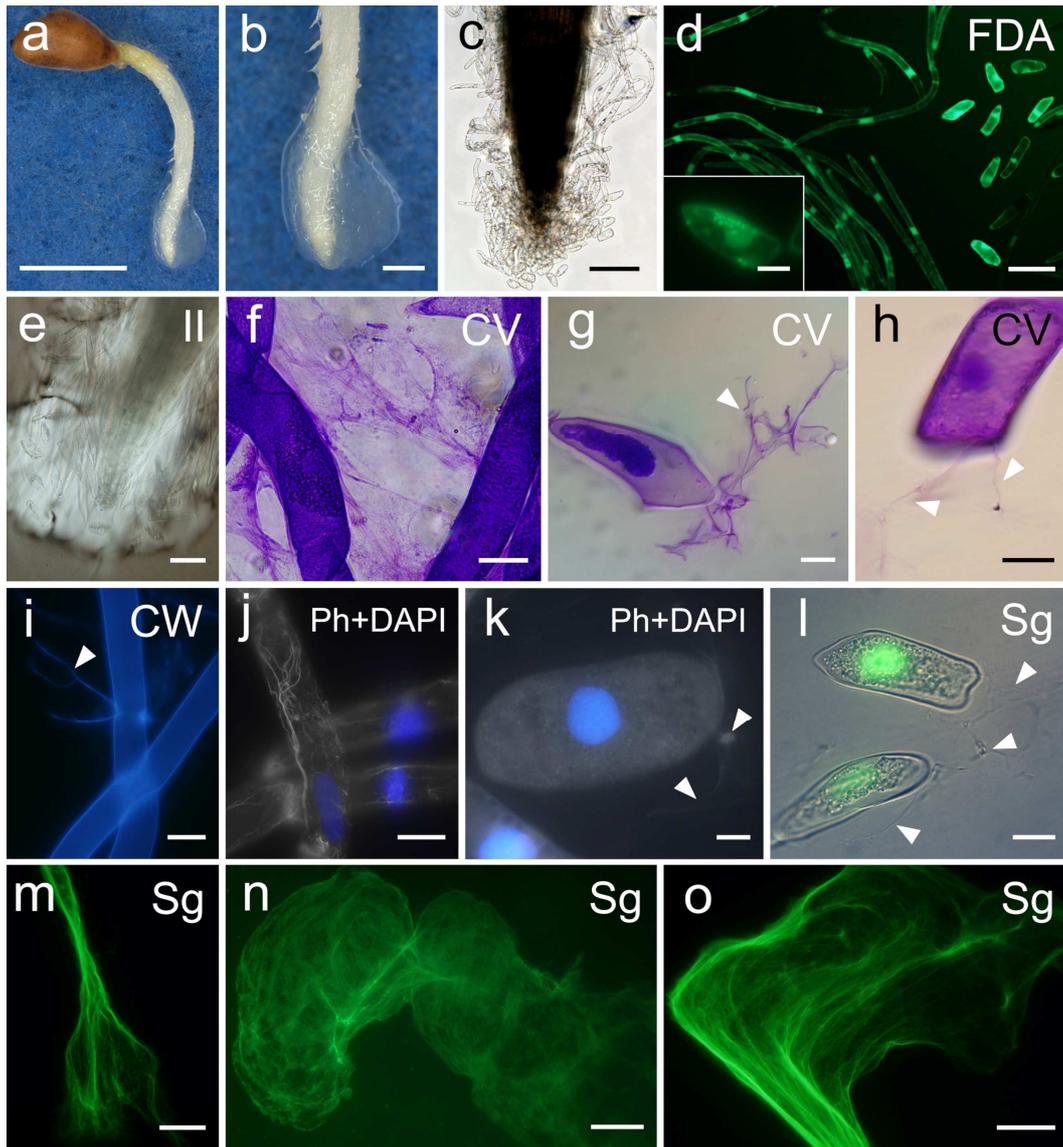
Fig. 1 Histological and histochemical imaging of the main components of the root extracellular traps in the early growth stage of *Pinus densiflora*. **a**, **b** A root tip after swelling treatment. **c** A root cap and root-associated, cap-derived cells (AC-DCs). **d** Cell viability of AC-DCs after detachment from the root cap. **e**, **f** Root mucilage secretions stained with India ink (**e**) and crystal violet (**f**) solutions. **g–i** Branched strands protruding from dead (**g**) and living (**h**, **i**) cells. **j**, **k** Merged images of actin filaments (gray) and nuclei (blue) in AC-DCs. **l** Nuclei of dead cells and strands protruding from cells. **m–o** Extracellular DNA spread in a thread-like or web-like structure. The abbreviations in the upper right corner of each image indicate the staining solutions applied: FDA, fluorescein diacetate solution; II, India ink; CV, crystal violet; CW, calcofluor white; Ph, Acti-stain 555 fluorescent phalloidin; DAPI, 4′,6-diamidino-2-phenylin-dole; and Sg, SYTOX Green. White arrowheads point to branched strands. The images in **c** and **l** were adjusted for brightness and contrast using GIMP. Bars: **a** = 5 mm; **b** = 1 mm; **c** = 200 µm; **d** (lower right), **e** = 100 µm; **n** = 50 µm; **d** (lower left), **f**, **h–j**, **l**, **m**, **o** = 20 µm; **g**, **k** = 10 µm
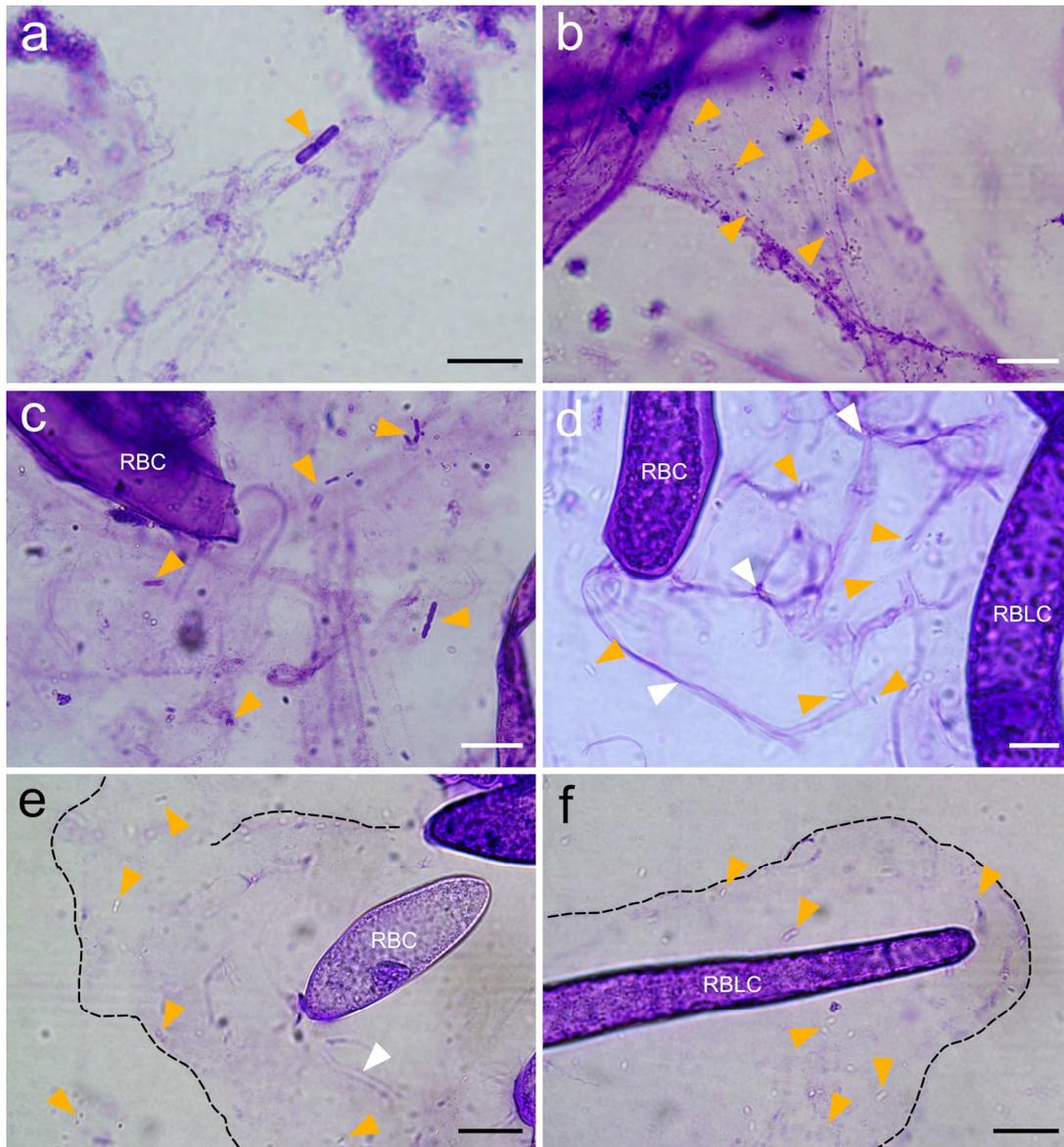
Fig. 2 Trapping of rhizobacteria by root mucilage secretions. The primary root and bacterial strains were co-incubated at 25℃ for 8 h (**a**, **b**) or 2 days (**c–f**). Staining with crystal violet solution revealed the results of co-incubation with **a**, **c** *Bacillus* sp. strain O-EM7 and **b**, **d–f** *Paraburkholderia* sp. strain O-NM9. RBC, root border cell; RBLC, root border-like cell. White and yellow arrowheads point to branched strands and bacterial cells, respectively. The black dashed lines in **e**, **f** denote the boundaries of the root mucilage. The images in **a**, **d–f** were adjusted for brightness and contrast using GIMP. Bars: **b**, **c**, **e**, **f** = 20 µm; **d** = 10 µm; **a** = 5 µm
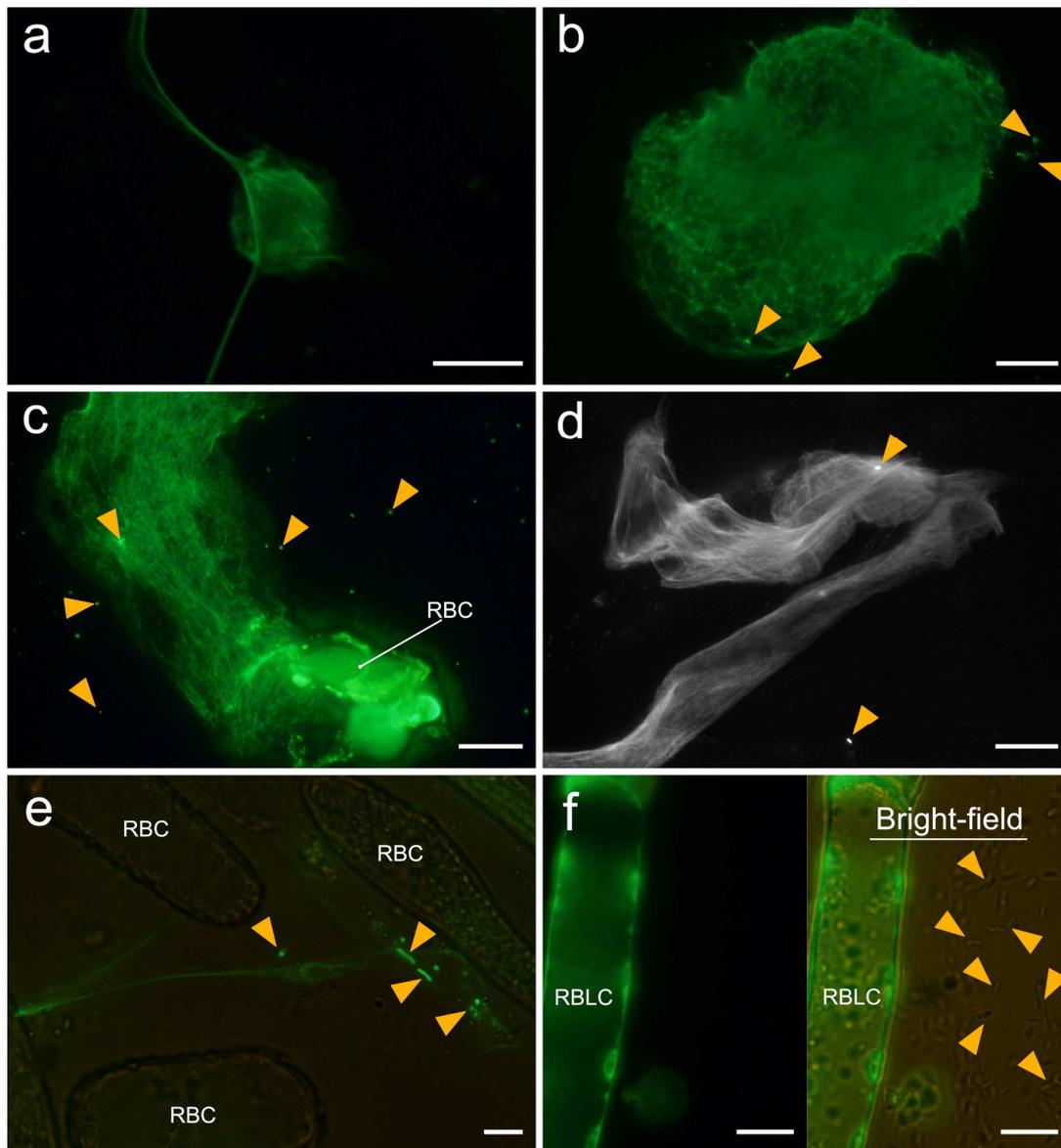
Fig. 3 Visualization of the unfolding of extracellular DNA and subsequent trapping of rhizobacteria. The primary root of *Pinus densiflora* and bacterial strains were co-incubated at 25°C for 8 h (**a**) or 2 days (**b**–**f**). Staining with SYTOX Green revealed the results of co-incubation with **a** *Paraburkholderia* sp. strain O-EM8; **b**, **e** *Bacillus* sp. strain O-EM7; and **c**, **d**, **f** *Paraburkholderia* sp. strain O-NM9. Yellow arrowheads point to dead (**a**–**e**) and live (**f**) bacterial cells. Bars: **a**, **c** = 50 µm; **b**, **d** = 20 µm; **e**, **f** = 10 µm
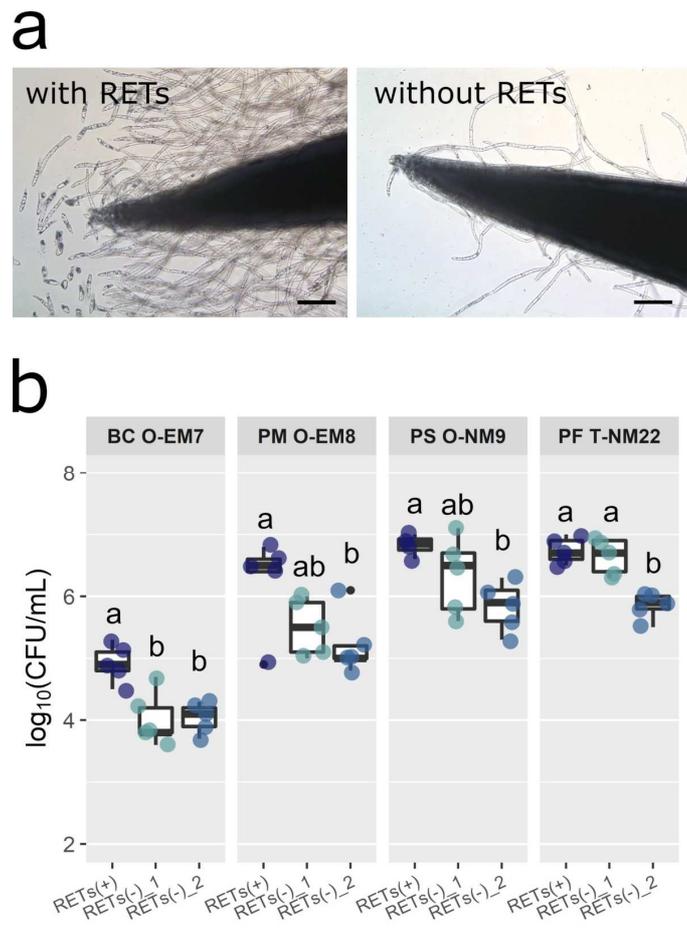
Fig. 4 a Primary root tip of *Pinus densiflora* before and after the root extracellular traps (RETs) were removed.

b Colony forming units in the rhizosphere of *P. densiflora* after 2 days of co-incubation with the rhizobacterial

strains (n = 5) *Bacillus* sp. strain O-EM7 (BC O-EM7), *Paraburkholderia* sp. strain O-EM8 (PM O-EM8),

Paraburkholderia sp. strain O-NM9 (PS O-NM9), and *Paraburkholderia* sp. strain T-NM22 (PF T-NM22). In

b, three treatments are compared: RETs (+), control treatment (no RET removal); RETs (–)_1, removal of

RETs before the co-incubation; and RETs (–)_2, removal of RETs after the co-incubation. Different letters (a,

b) indicate significant (p < 0.05) differences among the treatments according to Tukey's HSD test
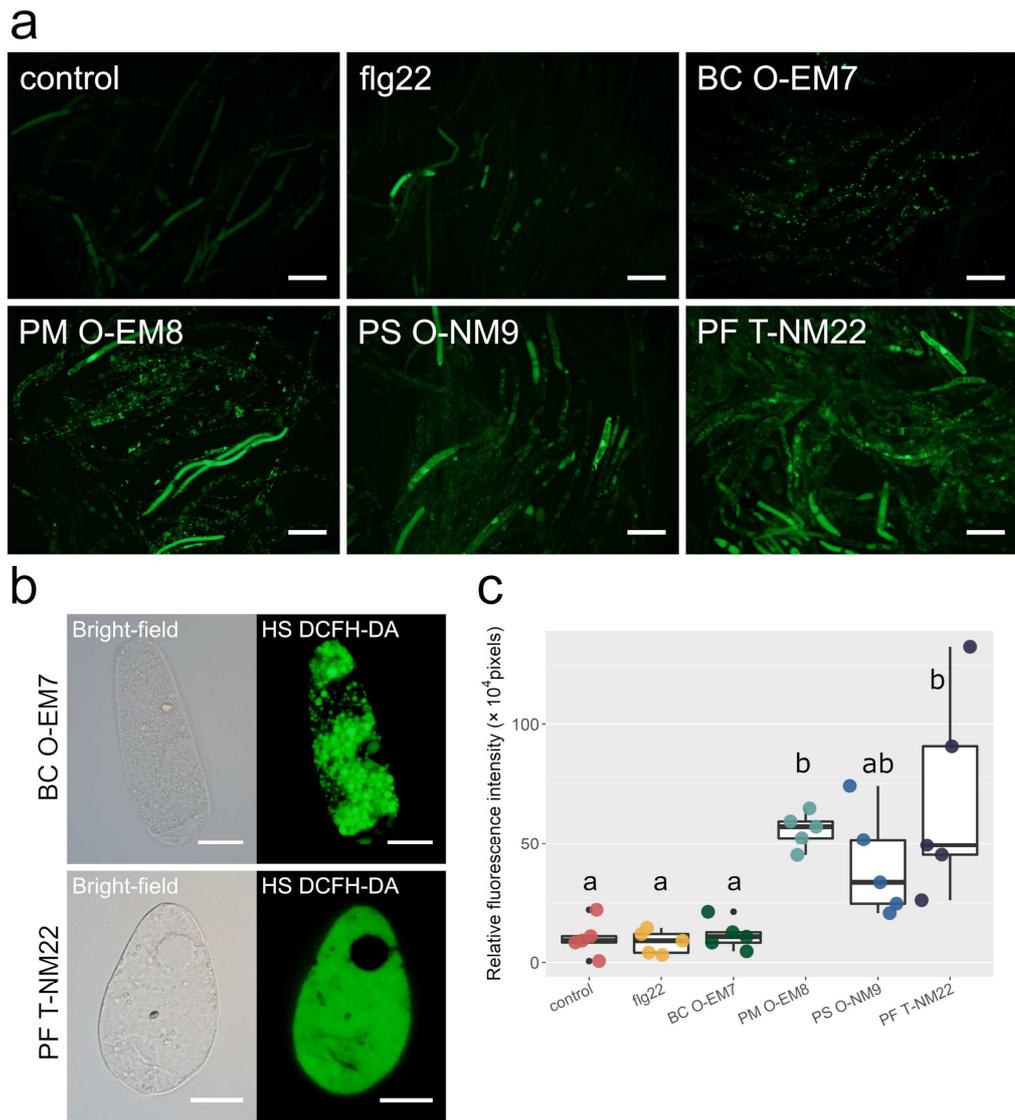
Fig. 5 a Production of reactive oxygen species (ROS) in root-associated, cap-derived cells (AC-DCs) from *Pinus densiflora* in the early growth stage after various treatments: Control, no treatment; flg22, incubation with the peptide flg22; BC O-EM7, incubation with *Bacillus* sp. strain O-EM7; PM O-EM8, incubation with *Paraburkholderia* sp. strain O-EM8; PS O-NM9, incubation with *Paraburkholderia* sp. strain O-NM9; PF T-NM22, incubation with *Paraburkholderia* sp. strain T-NM22. Bars = 100 µm. **b** Fluorescent patterns of root border cells that showed ROS bursts, detected using the fluorescent probe ROS Assay Kit – Highly Sensitive DCFH-DA (HS DCFH-DA). Bars = 20 µm. **c** Relative fluorescence intensity of total ROS in the AC-DCs from the early growth stage of *P. densiflora* in response to inoculation of each bacterial strain (n = 5). Different letters (a, b) indicate significant ($p < 0.05$) differences among the treatments and bacterial strains, according to Tukey's HSD test
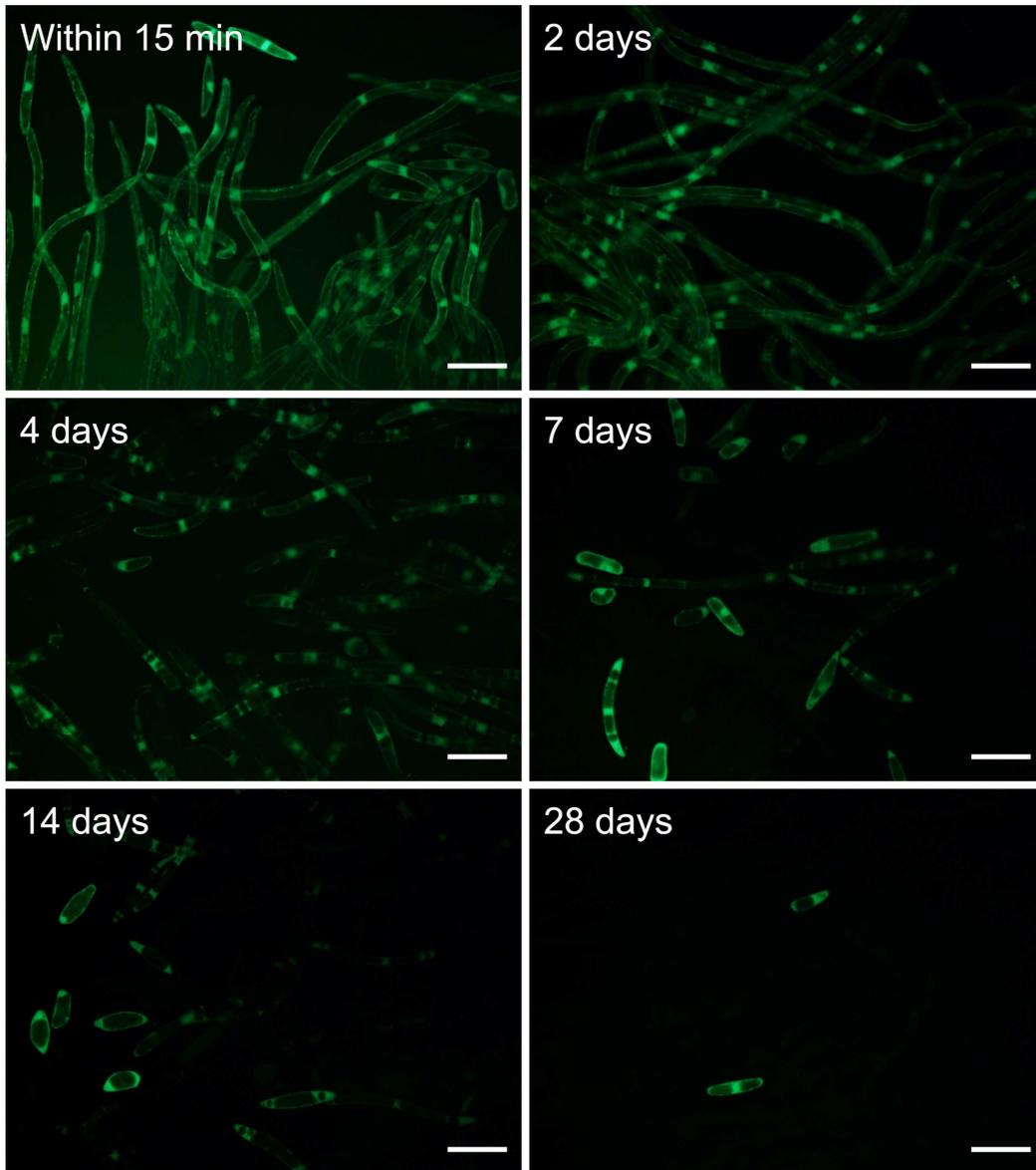
Fig. S1 Cell viability of root-associated, cap-derived cells (AC-DCs) from the early growth stage of *Pinus densiflora*. AC-DCs separated from the root cap were incubated in a 1.5 mL tube containing 200 μL sterile water at 25°C within 15 min and for 2, 4, 7, 14, and 28 days. To identify live versus dead cells, we used fluorescein diacetate solution (1 μg·mL^{-1} in phosphate-buffered saline) and 0.01% (v/v) Evans blue solution. Bars = 100 μm

742 **Captions for Online Resources** ※ **Not available in the preprint version**

743

744 The Online Resources (movies) were adjusted only for brightness and contrast using DaVinci Resolve

745 ver. 18.1.2 (https://www.blackmagicdesign.com/products/davinciresolve/), except for Online Resource 1

746

747 **Online Resource 1**

748 Release of root border cells from the root cap and expansion of root border-like cell in response to

749 affusion (sterile water)

750

751 **Online Resource 2**

752 Dispersion of root-associated, cap-derived cells and mucilage secretion in India ink solution

753

754 **Online Resource 3**

755 Cytoplasmic streaming of root-associated, cap-derived cells immediately after the detachment

756

757 **Online Resource 4**

758 Cytoplasmic streaming of root-associated, cap-derived cells after 7 days of incubation (part 1)

759

760 **Online Resource 5**

761 Cytoplasmic streaming of root-associated, cap-derived cells after 7 days of incubation (part 2)

762

763 **Online Resource 6**

764 Root mucilage encompassing the root-associated, cap-derived cells, with adhered rhizobacterial cells

765 (*Bacillus* sp. strain O-EM7)

766

767 **Online Resource 7**

768 Root mucilage encompassing the root-associated, cap-derived cells, with adhered rhizobacterial cells

769 (*Paraburkholderia* sp. strain O-NM9) (part 1)

770

771 **Online Resource 8**

772 Root mucilage encompassing the root-associated, cap-derived cells, with adhered rhizobacterial cells

773 (*Paraburkholderia* sp. strain O-NM9) (part 2)

774

775