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Title	Phenazine-1-carboxylic acid (PCA) produced by <i>Paraburkholderia phenazinium</i> CK-PC1 aids postgermination growth of <i>Xyris complanata</i> seedlings with germination induced by <i>Penicillium rolfsii</i> Y-1
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2 ***phenazinium CK-PC1* aids post-germination growth of *Xyris complanata***  
3 **seedlings with germination induced by *Penicillium rolfsii* Y-1**

4  
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25 **Phenazine-1-carboxylic acid (PCA) produced by *Paraburkholderia***  
26 ***phenazinium CK-PC1* aids post-germination growth of *Xyris complanata***  
27 **seedlings with germination induced by *Penicillium rolfsii* Y-1**

28

29

30 Abstract

31 Symbiosis of *Penicillium rolfsii* Y-1 is essential for the seed germination of Hawaii yellow-eyed grass  
32 (*Xyris complanata*). However, the local soil where the plants grow naturally often suppresses the radicle  
33 growth of the seedlings. This radicle growth was drastically restored by co-inoculation of  
34 *Paraburkholderia phenazinium* isolate CK-PC1, which is a rhizobacterium of *X. complanata*. It was  
35 found that the isolate CK-PC1 produced phenazine-1-carboxylic acid (PCA, **1**) as a major metabolite.  
36 The biological effects of PCA (**1**) were investigated using the seeds of *X. complanata* and mung bean  
37 (*Vigna radiata*), and it was uncovered that the symbiosis of the isolate CK-PC1 was essential for the  
38 post germination growth of *X. complanata* and the metabolite, PCA (**1**) might partially contribute to  
39 promote the growth of the plants.

40

41 Key words: phenazine-1-carboxylic acid; plant growth promoting activity; *Paraburkholderia*  
42 *phenazinium*.

43

#### 44 **Introduction**

45 Plant seeds are often dormant in soil, with seed dormancy leading to the formation of a  
46 large seed bank [1-3]. Strigolactones are well-studied chemical factors that break seed  
47 dormancy, such as the seeds of witchweed (*Striga* sp.), and *Orobanche* sp. [4]. In 2004,  
48 Flematti *et al.* reported the isolation and identification of karrikins, a group of novel dormant  
49 seed-breaking substances, from bush fire smoke and smoked soil [5, 6]. Karrikins were  
50 recognized by the dormant seeds of such fire-following plants, and they also accelerated  
51 *Arabidopsis* seed germination [7]. In addition, it was known that semi-symbiotic  
52 microorganisms such as *Rhizoctonia* in orchids and heaths involved to break seed dormancy  
53 [8, 9].

54 In previous study, Tamura *et al.* reported that symbiosis of *Penicillium rolfsii* Y-1 was  
55 essential for the seed germination of Hawaii yellow-eyed grass (*Xyris complanata*), whose  
56 seeds have also dormant property, although we could not show any mechanism for breaking  
57 seed dormancy of *X. complanata* [10]. In the course of continuous research, it was found that

58 *Penicillium* sp. Y-1 alone was not able to accomplish post germination growth. On the other  
59 hand, the germinated seeds of *X. complanata* exhibited better growth when the seedling was  
60 treated with a suspension extracted from non-sterilized local soil. It was hypothesized that other  
61 unidentified factors in the local soil are required for the post germination growth of *X.*  
62 *complanata*. In this paper, we reported that co-inoculation of *Paraburkholderia phenazinium*  
63 isolate CK-PC1, and the rhizobacterium of *X. complanata*, canceled the growth inhibitory  
64 effect of the local soil, and the metabolite of the strain CK-PC1, phenazine-1-carboxylic acid  
65 (PCA, **1**) might partially contribute to promote the growth of the plants.

66

## 67 **Materials and Methods**

68

### 69 ***General***

70 All reagents used were of analytical grade. FTIR spectra were recorded on an FT/IR-4100  
71 spectrometer (JASCO, Tokyo, Japan). NMR spectra were measured using an EX270  
72 spectrometer (JEOL, Tokyo, Japan). UV spectra were measured on a U-3310  
73 spectrophotometer (Hitachi, Tokyo, Japan). MS spectra were obtained using a JMS-T100GCV  
74 instrument (JEOL, Tokyo, Japan).

75

76 ***Plants, microorganisms and the local soil***

77 Seeds of *X. complanata* and the local soil were collected from the Kalamangan area,  
78 Central Kalimantan, Indonesia (2°17'S 114°01'E), in August 2016 and 2018 according to the  
79 legal process prescribed by Japanese and Indonesian governments. *Penicillium* sp. Y-1 was  
80 isolated from seeds of *X. complanata* according to the reported methods of Tamura *et al.* [10].  
81 Strain CK-PC1 was isolated from the root surface of the *X. complanata* seedlings, which were  
82 inoculated with a suspension of the non-sterilized local soil. The seeds of Mung bean (*Vigna*  
83 *radiata*) without fungicidal treatment were purchased from Nakahara Seed (Fukuoka, Japan).

84

85 ***Identification of fungus Penicillium rolfsii Y-1 by DNA analysis of the genomic ITS regions.***

86

87 The *Penicillium* sp. Y-1, a soil fungus that stimulates *X. complanata* seed germination [10],  
88 was identified via homology search for the DNA region between ITS-1 and ITS-4 at the species  
89 level. DNA sequences of the ITS regions between 5.8S and 18S rRNA genes were determined  
90 using the *ITS1 forward* (5'-TCC CGT AGG TGA ACC TGC GC-3') and *ITS4 reverse* (5'-TCC  
91 TCC GCT TAT TGA TAT GC-3') primers [11, 12]. The product was sequenced directly using

92 the BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA)  
93 with the same primers using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster  
94 City, CA) according to the manufacturer's protocol. The resulting DNA sequences were  
95 searched for homology using the BLASTN DNA searching system in DDBJ (Database of DNA  
96 Bank of Japan, Mishima, Japan) <<http://blast.ddbj.nig.ac.jp/blastn?lang=en>>.

97

#### 98 ***Phylogenetic analysis of Penicillium sp. Y-1***

99 In the phylogenetic analysis of the fungus, along with the microscopic observation of its  
100 conidium, a 472 bp sequence (accession no. AB277210.2) was analyzed using the maximum  
101 composite likelihood estimation, along with 11 reference species of *Penicillium* spp. and  
102 *Aspergillus oryzae*, *A. niger*, *A. costaricaensis*, *A. tamarii*, and *A. tabacinus*, all of which have  
103 been registered in the NCBI DNA database <<https://blast.ncbi.nlm.nih.gov/Blast.cgi>>. The  
104 neighbor-joining tree method was employed to identify the fungus at the species level using  
105 the computing tool for phylogenetic analysis in Molecular Evolutionary Genetics Analysis  
106 (MEGA v. 6.06) software [10, 13]. Clustering of the fungi was performed for further  
107 identification using an estimation of distances between all pairs of sequences with 1,000  
108 bootstrap replicates.

109

110 ***Cultivation of X. complanata.***

111 A conidial suspension of *P. rolfsii* Y-1 was used to stimulate seed germination of *X.*  
112 *complanata*. The fungus was grown on a potato dextrose agar (PDA) plate in the dark at 25°C  
113 for one week. A solution of sterile water (5 mL) was added to the plate, and the plates were  
114 slowly stirred to suspend conidia. The resulting conidial suspension was diluted 100-fold with  
115 sterile water. *P. phenazinium* CK-PC1 was cultured on a PDA plate in the dark at 30°C. After  
116 one week of cultivation, bacterial cells were suspended in sterile water and adjusted to OD 660  
117 nm = 1.0. The 200 µL of conidial suspension of *P. rolfsii* Y-1 and/or cell suspension of *P.*  
118 *phenazinium* CK-PC1 were added to sterilized local soil (20 mL) in a deep glass Petri dish (60  
119 mm diameter), on which soil at least 100 seeds of *X. complanata* were grown at 30°C under 12  
120 h light conditions with paneled LED light (20% blue and 80% red light) in a growth chamber  
121 (LTI-600 SD, Eyela, Tokyo, Japan) for 5 weeks.

122

123 ***Isolation of P. phenazinium CK-PC1.***

124 The strain CK-PC1 was isolated from the rhizosphere of *X. complanata* seedlings, which  
125 had been allowed to germinate by stimulating with *P. rolfsii* Y-1. On the sterilized local soil,

126 *X. complanata* seeds germinated but their radicle development was completely inhibited,  
127 resulting in seedling death within one to three months (Figure 1a). Conversely, the sterilized  
128 local soil was inoculated with 1 mL of the non-sterilized local soil suspension (1 mg soil/mL  
129 sterilized water), and the seedlings showed clear growth promotion and survival over 12  
130 months (Figure 1b).

131 Seedlings of *X. complanata* were grown on a sterilized local soil inoculated with a non-  
132 sterilized local soil suspension for ten months. From the root surface of 10 month-old *X.*  
133 *complanata* seedlings, root-associated microorganisms were isolated on modified  
134 Winogradsky's agar (MWA) plates [14]. Predominant bacterial colonies with 80-90%  
135 emergence frequency were isolated as a single colony (isolate CK-PC1) and further purified  
136 on MWA and PDA plates. DNA of the 16S rRNA gene region was amplified using PCR  
137 (AmpliTaq Gold 360 DNA polymerase, Applied Biosystems) on a Takara PCR Thermal Cycler  
138 Dice TP600 with its universal primer pair *27F/1525R* (5'-AGA GTT TGA TCC TGG CTC  
139 AG-3'/5'-AAA GGA GGT GAT CCA GCC-3'). The PCR conditions used were as follows:  
140 preheating at 95°C for 5 min, denaturation at 35 cycles at 95°C for 30 s, annealing at 60°C for  
141 30 s, extension at 72°C for 90 s, and completion at 72°C for 7 min. The second amplification  
142 of sequencing PCR used three forward (*27F*, *341F*, *1112F*) and three reverse (*803R*, *1080R*,

143 *and 1525R*) primers [15, 16, 17]. In the direct PCR sequence analysis, an ABI Prism 310  
144 genetic analyzer was used with a BigDye Terminator version 3.1 cycle sequencing ready-  
145 reaction kit (Applied Biosystems). Thus, the 16S rRNA gene sequence (DDBJ accession no.  
146 LC515237) was used for the homology search.

147

#### 148 ***Isolation of PCA (1) from the culture of P. phenazinium CK-PC1.***

149 To obtain the culture fluid, *P. phenazinium* CK-PC1 was cultured in potato dextrose broth  
150 media (2 L) for two weeks at 30°C with shaking at 100 rpm. After centrifugation, the culture  
151 fluid was adjusted to pH 4.0 with 1M HCl and extracted twice with the same volume of EtOAc.  
152 The combined organic layer was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated (159.1 mg). The  
153 residue was subjected to silica gel column chromatography (16 g Wakogel N-60) with *n*-  
154 hexane/CHCl<sub>3</sub>/MeOH at a ratio of 20:19:1 (20 mL of each ×10 fractions) to yield a yellowish  
155 solid (49.2 mg). The solid was recrystallized from acetone-hexane to yield yellow crystals (15.9  
156 mg).

157 Phenazine-1-carboxylic acid (PCA, **1**): mp 242-244°C. <sup>1</sup>H-NMR (270 MHz, CDCl<sub>3</sub>) δ: 8.97  
158 (1H, d, *J* = 7.0 Hz, H-2), 8.52 (1H, d, *J* = 8.6 Hz, H-4), 8.33 (1H, d, *J* = 8.6 Hz, H-9), 8.27 (1H,  
159 d, *J* = 8.0 Hz, H-6), 8.08-7.96 (3H, m, H-3, 7, 8). <sup>13</sup>C-NMR (66.5 MHz, CDCl<sub>3</sub>) δ: 164.9 (CO),

160 143.0, 142.3 (4a, 5a), 139.0, 138.8 (9a, 10a), 136.4 (2), 134.1 (4), 132.2 (7), 130.7 (8), 129.3  
161 (3), 129.0 (9), 126.9 (6), 123.9 (1). UV (CHCl<sub>3</sub>)  $\lambda_{\max}$  ( $\epsilon$ ): 252 (99600), 370 (19000). IR  $\nu_{\max}$   
162 cm<sup>-1</sup>: 3033, 2937, 2657, 175, 1471. HR-FD-MS [M]<sup>+</sup> calcd. for C<sub>13</sub>H<sub>8</sub>N<sub>2</sub>O<sub>2</sub>, 224.0586; found,  
163 224.0593. EI-MS *m/z* (rel. int, %), 224.1 [M]<sup>+</sup> (0.8), 180.0 (100), 178.9 (24), 152.9 (6.8), 125.9  
164 (2.4), 101.9 (4.1), 76.0 (5.3), 49.8 (6.2).

165

#### 166 ***Epicotyl gravitropism and epicotyl elongation assays using *V. radiata* seedlings***

167 Epicotyl gravitropism and epicotyl elongation assay were performed using *V. radiata*  
168 seedlings grown for 5 days at 25°C under a 16-h light photoperiodic condition. For the epicotyl  
169 elongation assay, the upper part of the plant (length: 5 mm) was removed and the stem (5 mm)  
170 below it was cut and used as epicotyl (Figure S1). An aqueous solution (2 mL) of 10  $\mu$ M IAA  
171 or PCA (**1**) was poured into a 2 cm glass petri dish, on which 10 epicotyl fragments were  
172 floated. After 16 h of incubation, the length of the epicotyl sections was measured using a ruler,  
173 and mean values were calculated with standard deviation. For the epicotyl gravitropism assay,  
174 the upper part of the plant (5 mm) was removed and the stem (length: 20 mm) below it was cut  
175 and used as epicotyl (Figure S1). On to a solidified plate (10 ml) containing 10  $\mu$ M IAA or  
176 PCA (**1**) using agar (1.5 %), 15 sections of the epicotyl were lied on the plate, and the plates

177 were left at 25 °C for 48 h in the dark making the bottom of the dish horizontal. Angles of  
178 epicotyl grown upward were measured as gravitropic responses using Image J ver. 1.51.

179

### 180 ***Measurement of seed germination rate and radicle growth using *V. radiata* seedlings***

181 For the measurement of germination rate, the surface-sterilized seeds of *V. radiata* were  
182 placed on a solidified plate (10 ml) using agar (0.3 %) containing 10 µM IAA, PCA (**1**), 1-  
183 naphthaleneacetic acid (NAA), 1-naphthoic acid (1-NTA), 2-naphthoic acid (2-NTA), 3-  
184 quinolinecarboxylic acid (3-QA), or 4-quinolinecarboxylic acid (4-QA) and stored at 25°C for  
185 36 h in the dark. Imbibed seeds in which the radicle broke the seed coat to develop were counted  
186 as germinated seeds. For the measurements of fresh weight and radicle length, the germinated  
187 seeds were treated with IAA, PCA (**1**) and the analogs, respectively, and were incubated for  
188 36 h in the same manner as described above.

189

### 190 ***Statistical analysis.***

191 All the data of biological assay and gene expression were statistically analyzed using one-  
192 way ANOVA followed by Dunnett's or Tukey's test.

193

194 **Results**

195

196 *Species-level identification of a fungus Penicillium rolfsii Y-1*

197 Our laboratory previously reported the isolation of a potent germination stimulating fungus,  
198 *Penicillium* sp. Y-1, from non-surface-sterilized seeds of Hawaii yellow-eyed grass (*X.*  
199 *complanata*), a monocot pioneer plant distributed throughout the degraded tropical peatland of  
200 Central Kalimantan in Indonesia [10]. However, species-level identification of the isolate  
201 *Penicillium* sp. Y-1 had not been performed yet. DNA sequence homology search for the ITS4  
202 region (accession number AB277210.2) of the genus *Penicillium* was performed combined  
203 with microscopic observation of its morphological characteristics, particularly at the  
204 conidiophore, as well as phylogenetic analysis using 11 different randomly selected species of  
205 the genera *Penicillium* and *Aspergillus* (Figure S2). In conclusion, *Penicillium* sp. Y-1 was  
206 identified as *Penicillium rolfsii* Y-1, the ITS region of which was recently registered in the  
207 NCBI GeneBank® database [18]. *P. rolfsii* is known to produce thermostable laminarinase and  
208 xylanase as a unique property among *Penicillium* fungi [18, 19]. Hence, this fungus can  
209 effectively degrade oil-palm trunk residue as if it were a brown rot fungus [20].

210

211 ***Restoration of radicle growth inhibition in X. complanata seedlings on humus-rich soil bed***  
212 ***by P. phenazinium CK-PC1***

213 As reported by Tamura *et al.*, *X. complanata* seeds sown on a sterilized local soil or boreal  
214 peat moss showed accelerated germination after inoculation with a conidial suspension of *P.*  
215 *rolfsii* Y-1 [10], although the post germination growth was completely suppressed in the soil  
216 (Figure 1a). On the other hand, treatments of *P. rolfisii* Y-1 together with a suspension of non-  
217 sterilized local soil from Central Kalimantan, Indonesia markedly promoted the growth of *X.*  
218 *complanata* seedlings (Figure 1b). It was hypothesized that a certain factor might cancel the  
219 unfavorable condition for the growth. In addition, strain CK-PC1 was isolated as a dominant  
220 bacterial colony with an appearance frequency of 80%–90% from the root surface of a well-  
221 grown *X. complanata* seedling. Using the 16S rRNA gene sequence for DDBJ homology, the  
222 CK-PC1 strain was identified as *Paraburkholderia phenazinium* (accession no. LC515237).

223 To address of the biological effects exhibited by co-inoculation of *P. rolfisii* Y-1 and *P.*  
224 *phenazinium* CK-PC1, the seeds of *X. complanata* were sown on the sterilized local soil. It was  
225 found that the radicles of the seedlings treated with *P. rolfisii* Y-1 alone hardly developed  
226 (Figure 1c), although better growth of roots and leaves were observed when *P. rolfisii* Y-1 and  
227 *P. phenazinium* CK-PC1 were co-inoculated (Figure 1d and 1e). The ability of *P. phenazinium*

228 CK-PC1 to stimulate seed germination was examined. However, the strain CK-PC1 did not  
229 promote the seed germination (Figure S3).

230

### 231 ***Metabolite produced by Paraburkholderia phenazinium CK-PC1***

232 As shown in Figure 1, *P. phenazinium* CK-PC1 restored seed growth of *X. complanata*  
233 that had been treated with germination stimulating fungus *P. rolfsii* Y-1 in the sterilized local  
234 soil. It was presumed that bioactive compounds produced by *P. phenazinium* CK-PC1 might  
235 be involved in the growth recovery.

236 *P. phenazinium* CK-PC1 was cultured in potato dextrose media (2L). According to the  
237 isolation procedure (Figure 2), phenazine-1-carboxylic acid (15.9 mg, PCA, **1**) was isolated as  
238 a major metabolite. The structure of **1** was determined based on MS, NMR, IR, and UV  
239 spectroscopic analyses and the comparison of those data with the spectral values in the  
240 literatures [21, 22] (Figures S4-S11).

241

### 242 ***Effects of phenazine-1-carboxylic acid (PCA, 1) on seedling growth***

243 The growth-promoting assay was conducted to investigate whether PCA (**1**) can replace  
244 the biological activity shown by the treatment of *P. phenazinium* CK-PC1. The *X. complanata*

245 seeds were treated with the strain Y-1 together with PCA (**1**) or the strain CK-PC1, and the  
246 treatment of the strain CK-PC1 and PCA (**1**) promoted the seedling growth of *X. complanata*,  
247 although the treatment of PCA (**1**) was less active than that of the strain CK-PC1 in growth  
248 promotion activity (Figure 3).

249 Since basic chemical structure of PCA (**1**) is a heterocyclic compound containing a  
250 carboxymethyl(ene) substituent, similar to that of IAA (Figure 4 a), it assumed that **1** might  
251 have a similar biological activity. To address the auxin-like activity of PCA (**1**), the effects of  
252 PCA (**1**) on auxin-regulated representative biological activities (Table 1) and auxin-responsive  
253 gene expressions (Figure S12) were examined using *V. radiata* seedlings. Auxin responsive  
254 genes, *IAA17* and *IAA20* in *V. radiata* were induced by treatment with IAA in root and shoot,  
255 although PCA (**1**) was able to induce the genes transcription only in the root, suggesting that  
256 PCA (**1**) might act like as IAA in root than in shoot (Figure S12). To further confirm the auxin-  
257 like activity of PCA (**1**), the biological impact of PCA (**1**) upon *Arabidopsis thaliana* was  
258 examined. Similar to IAA, PCA (**1**) strongly inhibited root elongation of *A. thaliana* (Figure  
259 S13), indicating that PCA (**1**) also has auxin-like activity in plant other than *V. radiata*. In  
260 addition, several additional analogs, bi- (or tri-) cyclic carboxylic acid compounds, were  
261 subjected to the seed germination and growth assays of *V. radiata*. Ten micromolar solution of

262 1-naphthaleneacetic acid (NAA), 1-naphthoic acid (1-NTA) and 2-naphthoic acid (2-NTA),  
263 and 3-quinolinecarboxylic acid (3-QA) and 4-quinolinecarboxylic acid (4-QA) were used to  
264 assess seed germination rate, fresh weight, and radicle elongation (Figure 4c, Figure S14). All  
265 of the compounds at 10  $\mu$ M effectively reduced the germination rate in a time-dependent  
266 manner until 24 h (Figure S14). Auxins, IAA and NAA showed potent inhibitory activity on  
267 radicle elongation among seven compounds. Based on these results, it was suggested that PCA  
268 (1) had weak but significant activity similar to IAA upon the root part.

269

## 270 **Discussion**

271

### 272 ***P. phenazinium* CK-PC1 promotes post germination growth of *X. complanata***

273 *P. rolfsii* Y-1 stimulated seeds germination of *X. complanata*, however, the seedlings  
274 subsequently showed poor radicle growth (Figure 1c) and were not able to grow for long  
275 periods (Figure 1a). On the other hand, co-inoculation of *P. rolfsii* Y-1 with *P. phenazinium*  
276 CK-PC1, which was isolated as a rhizobacterium of *X. complanata*, restored the radicle growth  
277 inhibition (Figure 1d), and supported clear radicle elongation and development to sustain the

278 long-term growth of *X. complanata* (Figure 1b). Furthermore, it was found that *P. phenazinium*  
279 CK-PC1 produced PCA (**1**), which is structurally similar to IAA, as a major metabolite.

280 It was generally accepted that auxin regulates root development [23]. Exogenous  
281 treatments of auxin inhibit primary root elongation and promote lateral root formation,  
282 affecting entire root architecture. The strain CK-PC1 might affect root development by  
283 modulating auxin responses in *X. complanata* root via PCA (**1**), ultimately leading to the  
284 promotion of root growth. PCA (**1**) has been reported to have many biological activities,  
285 including antibiotic activity, bacterial biofilm formation-inducing factor activity, and  
286 disturbance of the reactive oxygen species-quenching systems in phytopathogenic bacteria [24,  
287 25]. However, auxin-like activity of PCA (**1**) have not been reported so far.

288

### 289 ***PCA (1) and auxin exhibit similar activities on radicles growth***

290 Because of the structural similarity between IAA and PCA (**1**) (Figure 4a), it was  
291 supposed that PCA (**1**) might have auxin agonistic or antagonistic activities. Results of auxin  
292 assays using *V. radiata* (Table 1, Figure S14) indicated that PCA (**1**) had weak IAA-like  
293 activity, such as activities of epicotyl gravitropism, epicotyl elongation, germination and  
294 growth. Especially, PCA (**1**) enhanced the expression levels of some early auxin-response

295 genes in the roots of *V. radiata*. The *IAA20* expression level significantly increased  
296 (approximately 50-fold upregulation) in the radicles of *V. radiata* (Figure S12). Since it was  
297 reported that *IAA20* belonged to Aux/IAA repressor protein interacting with Auxin Responsive  
298 Factor (ARF) transcriptional activator to regulate auxin-responsive gene expression [26],  
299 PCA (**1**) would acts as an auxin-like substance. However, the effects of PCA (**1**) are not  
300 completely comparable to those of IAA (Figure S14), because PCA (**1**) was more potent in  
301 roots than in shoots, and not able to increase the transcriptional levels of representative auxin  
302 response genes other than *IAA20* (Figure S14). In addition to *V. radiata*, 10  $\mu$ M PCA (**1**)  
303 showed a similar inhibitory effect on radicle elongation of *A. thaliana* seedlings (Figure S13).  
304 The result indicated the potential for PCA (**1**) to act as an IAA-like substance in other plants.  
305 Regard to a weak auxin-like activity of PCA (**1**), further studies should be done for other kinds  
306 of the responsive genes such as those for the signaling, transport and metabolisms.

307

308 ***PCA (1) would be important bioactive metabolite for the plant growth-promoting***  
309 ***rhizobacterium***

310 In this report, it was found that PCA-producing *P. phenazinium* CK-PC1 was necessary  
311 for normal growth and survival for the pioneer plant *X. complanata* in the humic substance-

312 rich, tropical woody peatland such as deforested open areas. Indeed, PCA-producing  
313 *Burkholderia* spp. or *Pseudomonas* spp. have often been isolated as a plant growth-promoting  
314 rhizobacterium (PGPR) [27- 29].

315 Genes associated with PCA (**1**) biosynthesis have also been investigated in PCA-  
316 producing bacteria [30-32]. PCA-producing bacterial communities in the rhizospheres of some  
317 crop and environmental samples were assessed by metagenomics analysis using some PCA (**1**)  
318 biosynthetic genes as marker. In the other environmental samples, it was revealed that many  
319 marine bacteria have the potential to produce PCA (**1**) and some other phenazine carboxylates,  
320 which implied that the products was synthesized beyond classes of Proteobacteria [33].

321 PCA (**1**) has long been thought to act as an antibiotic to suppress the virulence and  
322 population size of soil-borne phytopathogens, including *Fusarium* and *Alternaria* fungi [34].  
323 However, our study strongly suggested that PCA (**1**) was an auxin-like substance with  
324 particular functions in the underground parts of plants. Thus, interactions between native  
325 pioneer plants and some soil microorganisms, including PCA-producing bacteria, may indicate  
326 the existence of new soil ecosystems, which encourage to do further research to uncover the  
327 unknown crosstalk between plant roots and rhizomicroorganisms.

328

329

330 **Author contributions**

331 Y. Hashidoko, H.C. Wijaya, and Y.A. Nyon conceived and designed the experiments. M.

332 Hane, Y. Sakihama, M. Hashimoto, and H. Matsuura performed the essential experiments and

333 analyzed the data. M. Hane, Y. Hashidoko, Y. Sakihama, and M. Hashimoto wrote the paper.

334 All authors discussed the results and commented on the manuscript.

335

336 **Disclosure statement**

337 No potential conflict of interest was reported by the authors.

338

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- 426

427 Table 1. Auxin-like activity of PCA (1) against *V. radiate* compared to IAA

428 The concentration of PCA (1) and IAA was 10  $\mu$ M.

	Epicotyl gravitropism ( $^{\circ}$ ) ( $n=15$ )	Epicotyl length (mm) ( $n=30$ )	Germination rate (%) ( $n=100\times 3$ )	Fresh weight (mg) ( $n=75$ )	Radicle length (mm) ( $n=15$ )
Control <sup>a)</sup>	15 $\pm$ 3	6 $\pm$ 1	62.6 $\pm$ 8.3	236.8 $\pm$ 5.3	26.3 $\pm$ 0.8
PCA	29 $\pm$ 3*	11 $\pm$ 1***	37.3 $\pm$ 4.6**	201.3 $\pm$ 4.3***	17.4 $\pm$ 0.6***
IAA	34 $\pm$ 2***	12 $\pm$ 1***	21.3 $\pm$ 2.3***	202.1 $\pm$ 4.2***	13.8 $\pm$ 0.3***

429 a) Control plants were treated with RO water instead of PCA (1) and IAA solution.

430 Dunnett's test \*:  $p<0.05$ , \*\*:  $p<0.01$ , \*\*\* :  $p<0.001$

431

432 **Figure captions**

433

434 **Figure 1.** Restoration of *X. complanata* growth by co-inoculation with a rhizobacterium, *P.*

435 *phenazinium* CK-PC1 from non-sterilized local soil.

436 The seedlings of *X. complanata* were cultivated for 5 weeks on sterilized local soil.

437 (a) Seedlings of *X. complanata* treated with germination stimulating fungus *P. rolfsii* Y-1. (b)

438 Seedlings of *X. complanata* co-inoculated with germination stimulating fungus *P. rolfsii* Y-1

439 and the suspension of non-sterilized local soil. (c) Roots of *X. complanata* seedlings with *P.*

440 *rolfsii* Y-1. (d) Roots of *X. complanata* seedlings with *P. rolfsii* Y-1 + *P. phenazinium* CK-

441 PC1. Arrows indicate radicles. (e) Growth promotion of *X. complanata* seedlings by *P. rolfsii*

442 Y-1 and *P. phenazinium* CK-PC1. Seedlings germinated without microbial inoculation were

443 used as control. The leaf length of the seedlings cultivated for five weeks were measured. Each

444 value is represented by the mean  $\pm$  SD. Number of replicates were shown in the Figure 1e.

445 Dunnett's test, \*\*\*: $p < 0.001$ .

446

447 **Figure 2.** Isolation procedure for compound **1** from *P. phenazinium* CK-PC1.

448 a) indicating chemical structure of phenazine-1-carboxylic acid (PCA, **1**).

449

450 **Figure 3.** Growth promoting assay for PCA (**1**) purified from *P. phenazinium* CK-PC1 against  
451 *X. complanata* seedlings.

452 Germination of *X. complanata* seeds was stimulated by inoculation with *P. rolfsii* Y-1 on  
453 sterilized peat moss. Three-week-old seedlings were treated with 0.5  $\mu\text{mol}$  PCA (**1**)/20 mL soil  
454 or strain CK-PC1 and grown for an additional two weeks. Each value is represented by the  
455 mean  $\pm$  SD. Number of replicates were shown in the Figure 3. Tukey's HSD was used to  
456 compare the test intervals, showing statistically significant differences between different letters  
457 ( $p < 0.05$ ).

458

459 **Figure 4.** Biological activities of bi-(or tri-) cyclic carboxylic acids upon *V. radiata* seedlings.

460 (a) Semi-superimposed projection of PCA (**1**) and IAA. (b) Structures of bi-(or tri-) cyclic  
461 carboxylic acids. (c) Effects of bi-(or tri-) cyclic carboxylic acids on *V. radiata* growth. *V.*  
462 *radiata* seeds were cultured in the dark at 25°C for 36 h on 0.3% agar plate (control) and 0.3%  
463 agar plate containing 10  $\mu\text{M}$  of each bi-(or tri-) cyclic carboxylic acid. Each value is  
464 represented by the mean  $\pm$  SD. Number of replicates were  $n = 75$ . Dunnett's test, \*:  $p < 0.05$ ,

465 \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ . Tukey's HSD was used to compare the test intervals, showing  
466 statistically significant differences between different letters ( $p < 0.05$ ).

467

468 **Graphical abstract caption**

469 Restoration of *Xyris complanata* seedlings growth by co-inoculation of *Penicillium rolfsii* Y-  
470 1 and *Paraburkholderia phenazinium* CK-PC1.

471

472

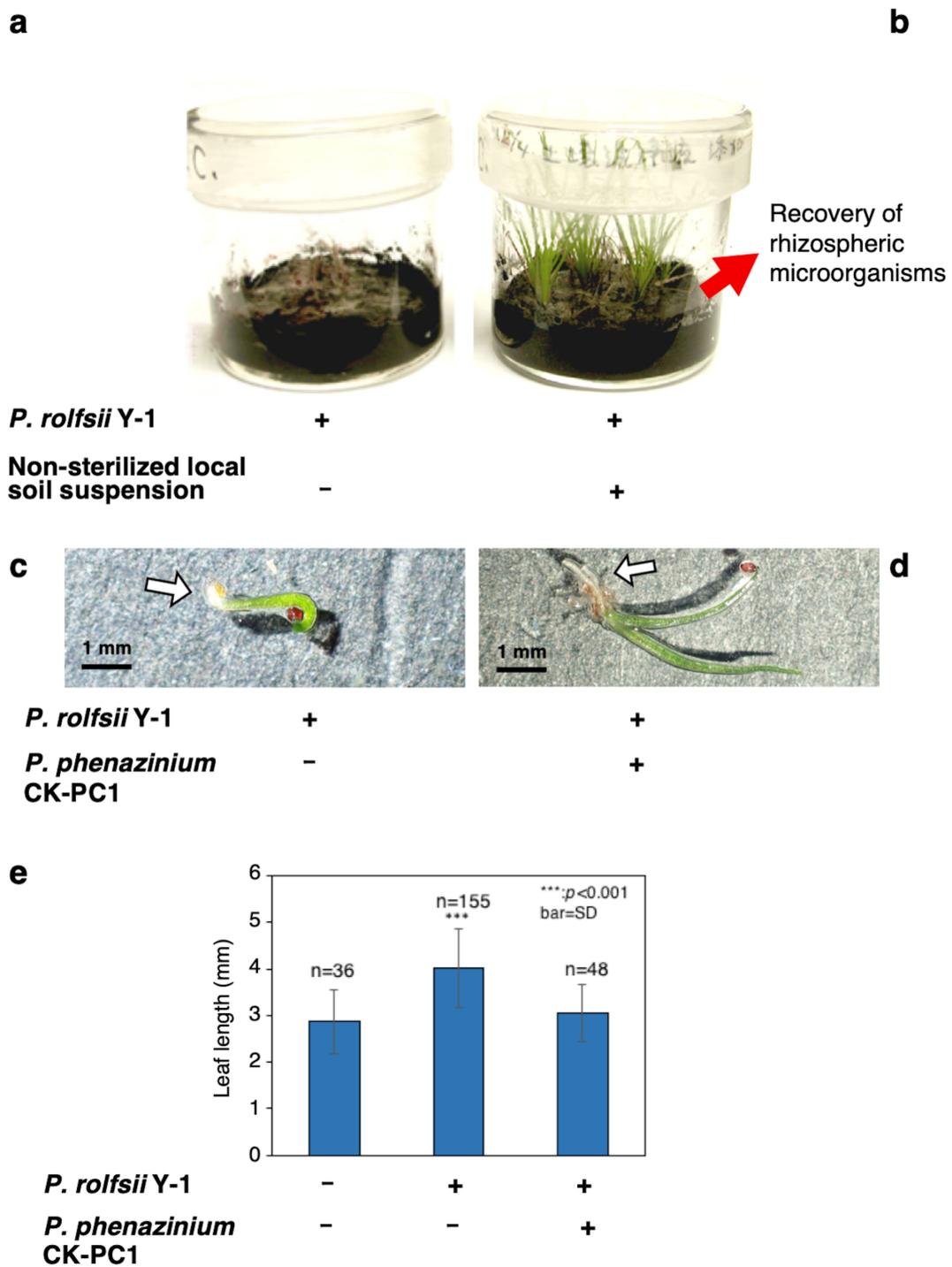


Figure 1

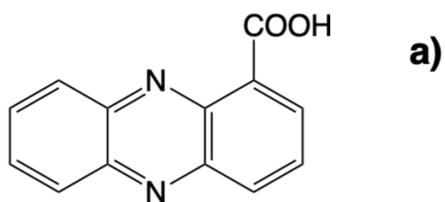
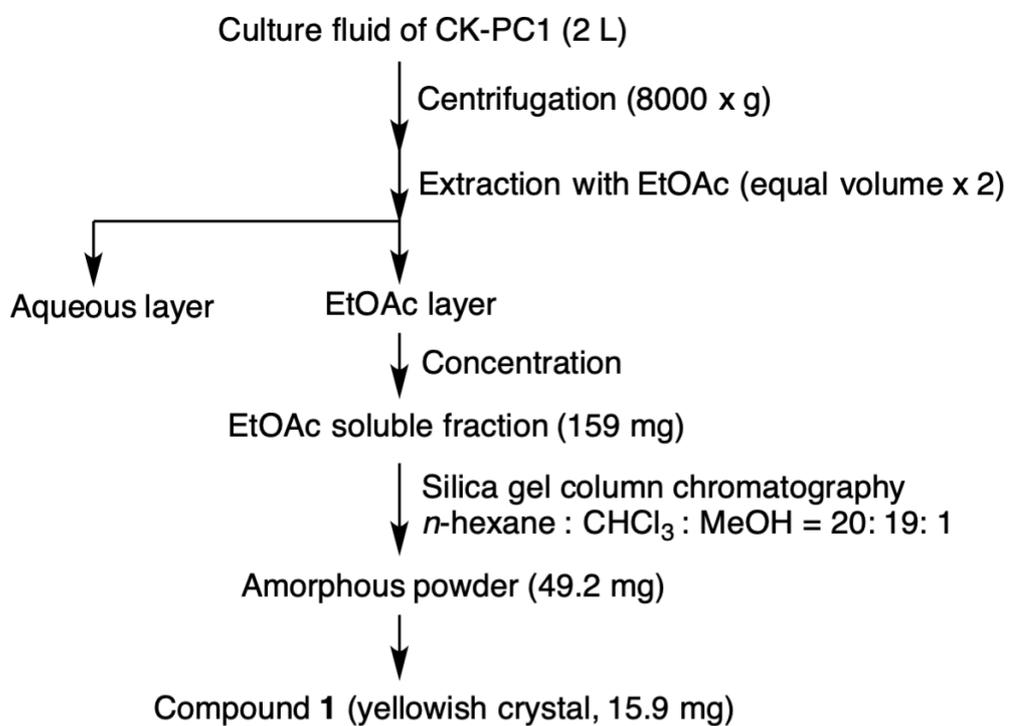


Figure 2

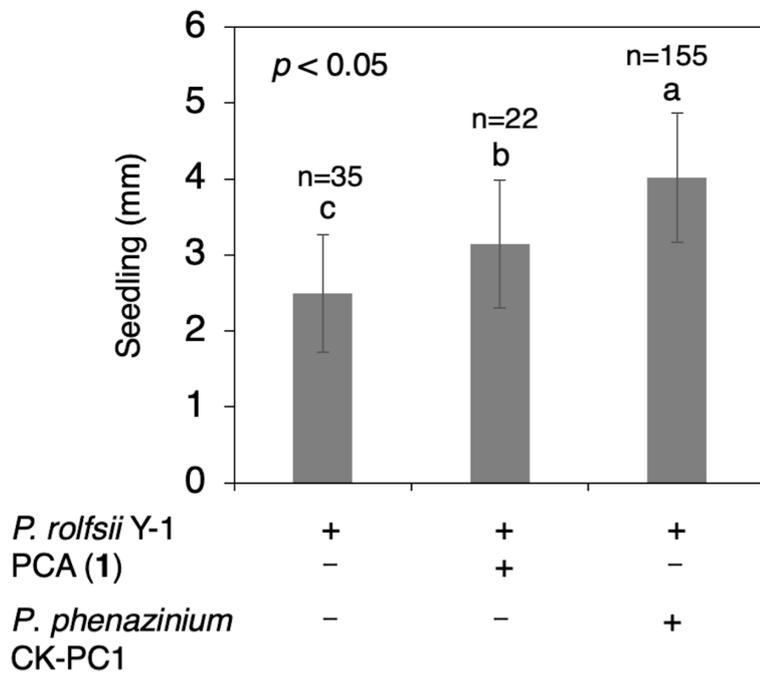


Figure 3

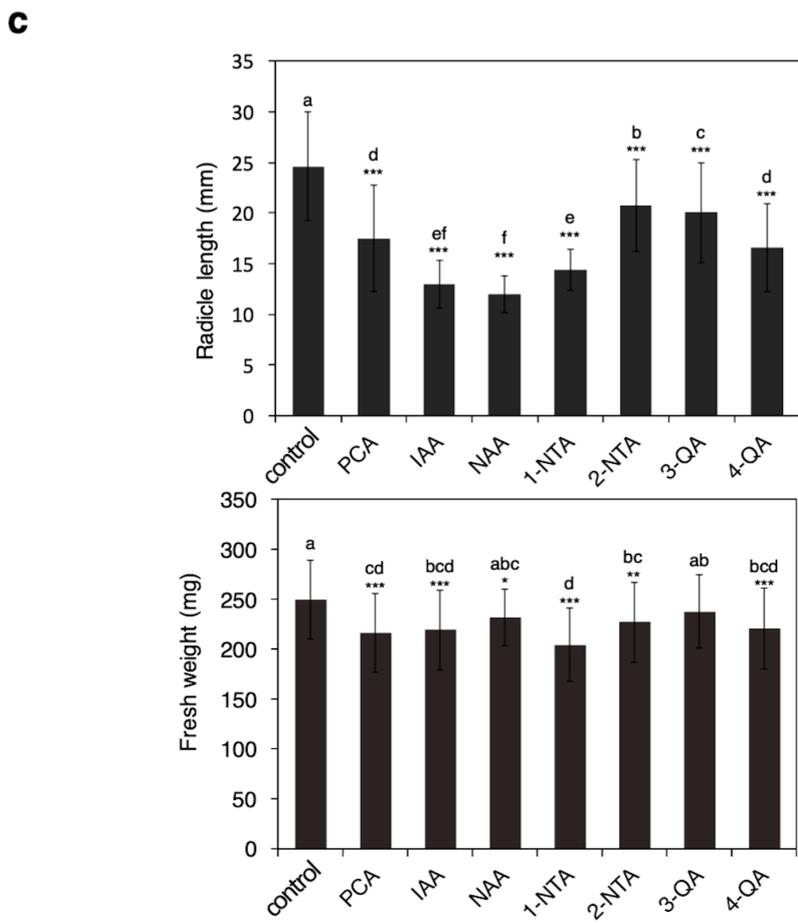
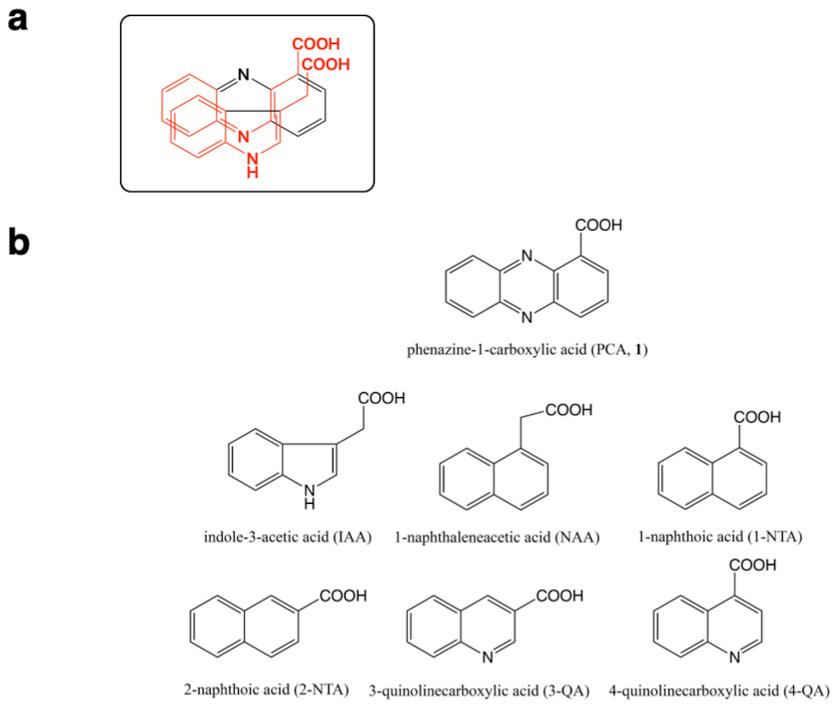


Figure 4

## Supporting information

**Phenazine-1-carboxylic acid (PCA) produced by *Paraburkholderia phenazinium CK-PC1* aids post-germination growth of *Xyris complanata* seedlings with germination induced by *Penicillium rolsii***

**Y-1**

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### Materials and methods

#### *Germination assay of V. radiata.*

Germination rate was measured as follows: 25 surface-sterilized *V. radiata* seeds were placed on a 10 mL portion of 10  $\mu$ M IAA, PCA, 1-naphthaleneacetic acid (NAA), 1-naphthoic acid (1-NTA), 2-naphthoic acid (2-NTA), 3-quinolinecarboxylic acid (3-QA), or 4-quinolinecarboxylic acid (4-QA)-containing 0.3% agar plates and stored at 25°C for 36 h in the dark. Imbibed seeds in which the radicle broke the seed coat to develop were

counted as germinated seeds. Next, 0.3% agar was used as a control. The germination rate was measured every 3 h for 36 h. This experiment was performed in triplicates.

### ***PCA (I) or IAA treatment of *V. radiata* seedlings for RNA extraction***

After imbibition of the dry seeds and growth at 25°C under 16-h lighting conditions, 5-day-old *V. radiata* seedlings were harvested and separated into epicotyls, hypocotyls, radicles, cotyledons, and the first leaves using a sterile blade (Figure S1). Treatment of epicotyls and radicles (ten specimens each) was conducted as follows. Plant tissues were soaked in 5 mm deep 10 µM IAA solution or PCA (in 50 mM potassium phosphate buffer, pH 7.0) in glass Petri dishes, modifying the method described in a report by Yamamoto and colleagues [1]. Ten epicotyls (approximately 100 mg in fresh weight) and ten radicles (approximately 100 mg in fresh weight) were incubated in the test solution for 8 h at 30°C in the dark with slow rolling. At this point, plant tissues were recovered and rinsed with ice-cold 50 mM phosphate buffer followed by immediate RNA extraction.

### ***Total RNA extraction from *V. radiata* seedlings and cDNA synthesis***

Total RNA was extracted from the plant tissues crushed in liquid N<sub>2</sub> using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Then, 500 µL of RLT buffer and 5 µL of 2-mercaptoethanol were added to the crushed plant sample, and total RNA was extracted according to the manufacturer's protocol. RNase-free DNase I solution was added to the total RNA solution (30 µL), and the sample was incubated for 1 h at room temperature to

completely remove genomic DNA. The resulting RNA was precipitated in the solution after mixing with 5  $\mu$ L of 3 M sodium acetate and 125  $\mu$ L of 95% EtOH and allowed to stand overnight at  $-30^{\circ}\text{C}$ . Next, the solution was centrifuged at 14,000 rpm at room temperature, and the supernatant-removed precipitates were washed once with 625  $\mu$ L 70% EtOH. The resulting solution was centrifuged at 14,000 rpm for 10 min to obtain the precipitated RNA sample. After drying, the resulting total RNA precipitate was re-dissolved in 30  $\mu$ L RNase-free water. The 0.5  $\mu$ g sample of total RNA was reverse transcribed using a Takara PrimeScript RT Reagent Kit (Takara Bio, Ohtsu, Japan) with oligo-dT primers. The RT reactions were performed at  $37^{\circ}\text{C}$  for 15 min,  $85^{\circ}\text{C}$  for 5 s, and cooled to  $8^{\circ}\text{C}$ .

### ***Quantitative real-time PCR analysis***

All of the gene primers used for RT-qPCR for the targeted genes were designed and self-checked as original primers except for house-keeping genes (Table S1). Primers designed for some representative genes that are induced via auxin (auxin-induced genes) or respond to auxin through either up-regulation or down-regulation (auxin-responsive genes) were assayed using RT-qPCR to quantify their transcriptional level. The primer pairs *IAA17-1f/IAA17-1r* (5'-AGC TCC TTA TCT AAG AAA AGT GGA T-3'/5'-CAT CAG TTT CCT CTC ATT CAT-3',  $T_m$  60.9/58.7  $^{\circ}\text{C}$ ), *AUX28-2f/AUX28-2r* (5'-ATG TTC AGC TCC TTC ACC-3'/5'-ATC CAG TCA CCA TCC TTG TC-3',  $T_m$  58.3/62.0  $^{\circ}\text{C}$ ), and *IAA20-1f/IAA20-1r* (5'-GTG AAG GTC TAC ATG GAA GG-3'/5'-ATT CAT CTC TGT CCC CCA-3',  $T_m$  59.1/61.0 $^{\circ}\text{C}$ ) yielded PCR product fragment sizes

of 117, 135, and 108 bp, respectively. As the house-keeping gene, the SKP-1/ASK-interacting protein 16 gene was selected with the primer set *Skip16-f/Skip16-r* (5'-CAC CAG GAT GCA AAA GTG G-3'/5'-ATC CGC TTG TCC CTT GAA C-3',  $T_m$  64.6/65.5 °C) to yield 163 bp [2].

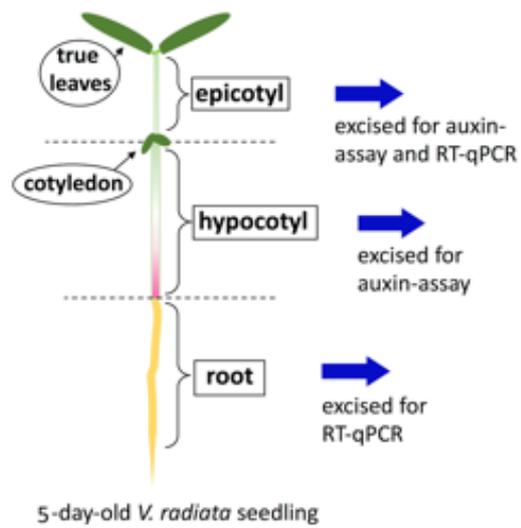
The auxin-responsive gene-targeting RT-qPCR was performed with the Thermal Cycler Dice Real Time System TP800 (Takara Bio, Ohtsu, Japan) using a SYBR Premix Ex Taq II (Tli RNaseH Plus, Takara Bio), cDNA corresponding to 50 ng of total RNA, and each specific primer pair in a 25  $\mu$ L reaction mixture. The following thermal conditions were used: denaturation at 95°C for 30 s, 40 cycles at 95°C for 5 s, and 60°C for 30 s. A dissociation curve analysis was performed at 60°C for 15 s, 60°C for 30 s, and 95°C for 15 s.

### ***Effect of PCA on Arabidopsis thaliana seed germination***

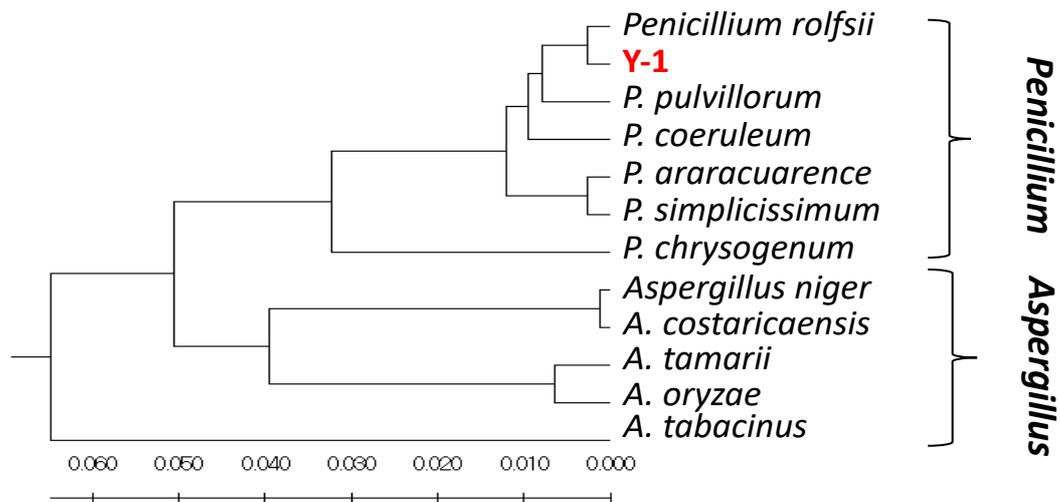
*Arabidopsis thaliana* seeds are often used for auxin assay, showing root growth inhibition as a response to auxin [3, 4]. 1/2 Murashige and Skoog Plant Salt Mixture and 1% sucrose (100 mL, pH 5.8) solidified with 1% gellan gum was kept at 60°C before adding 50  $\mu$ L of 10 mM IAA or PCA (**1**) in acetone, mixing well, and casting on plastic petri dishes (50 mL/dish). The control contained only 0.1% acetone in 1/2 MS solution. The prepared assay plates contained 10  $\mu$ M IAA or PCA (**1**), *A. thaliana* seeds were added horizontally, and the plates were set perpendicularly in a growth chamber and incubated at 22°C for 7 days under 16-h lighting conditions.

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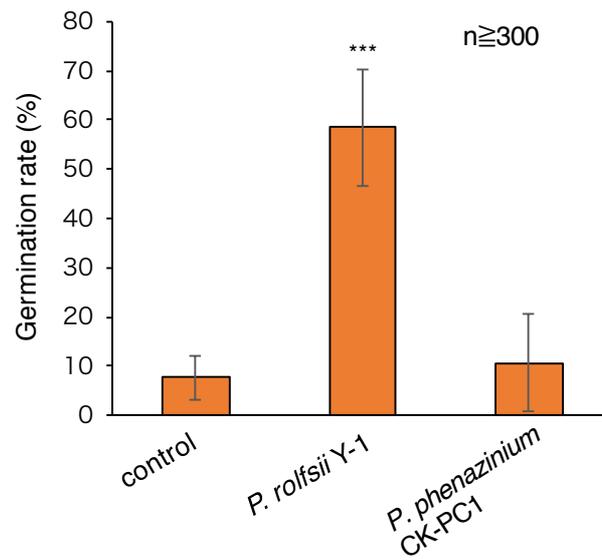


**Figure S1.** Names of each part in *V. radiata* seedlings.

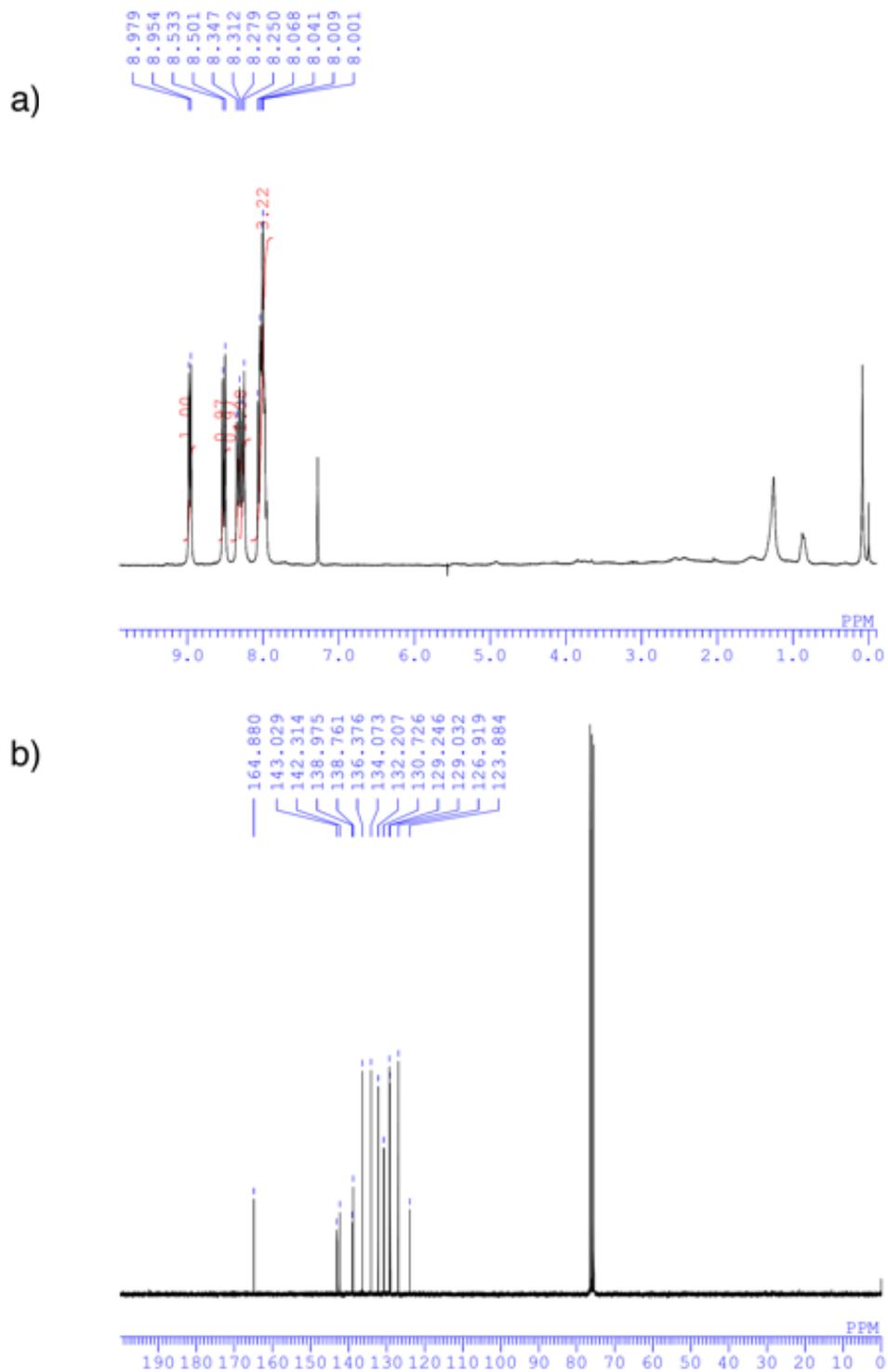


**Figure S2.** Phylogenetic analysis of *Penicillium* sp. Y-1 strain using ITS1-ITS4 region. The phylogenetic relationship of *Penicillium* sp. Y-1 strain was computed, along with 11 reference species of *Penicillium* and *Aspergillus* fungi selected from several groups of the fungi imperfecti. This neighbor-joining tree was deduced based on the ITS region between ITS1 and ITS4 obtained as 470-500 bp in size using the maximum composite likelihood method for the estimation of distances between all pairs of sequences simultaneously with 1,000 bootstrap replicates by the Kimura 2-parameter model. The scale bar represents 0.01 substitutions per nucleotide site.

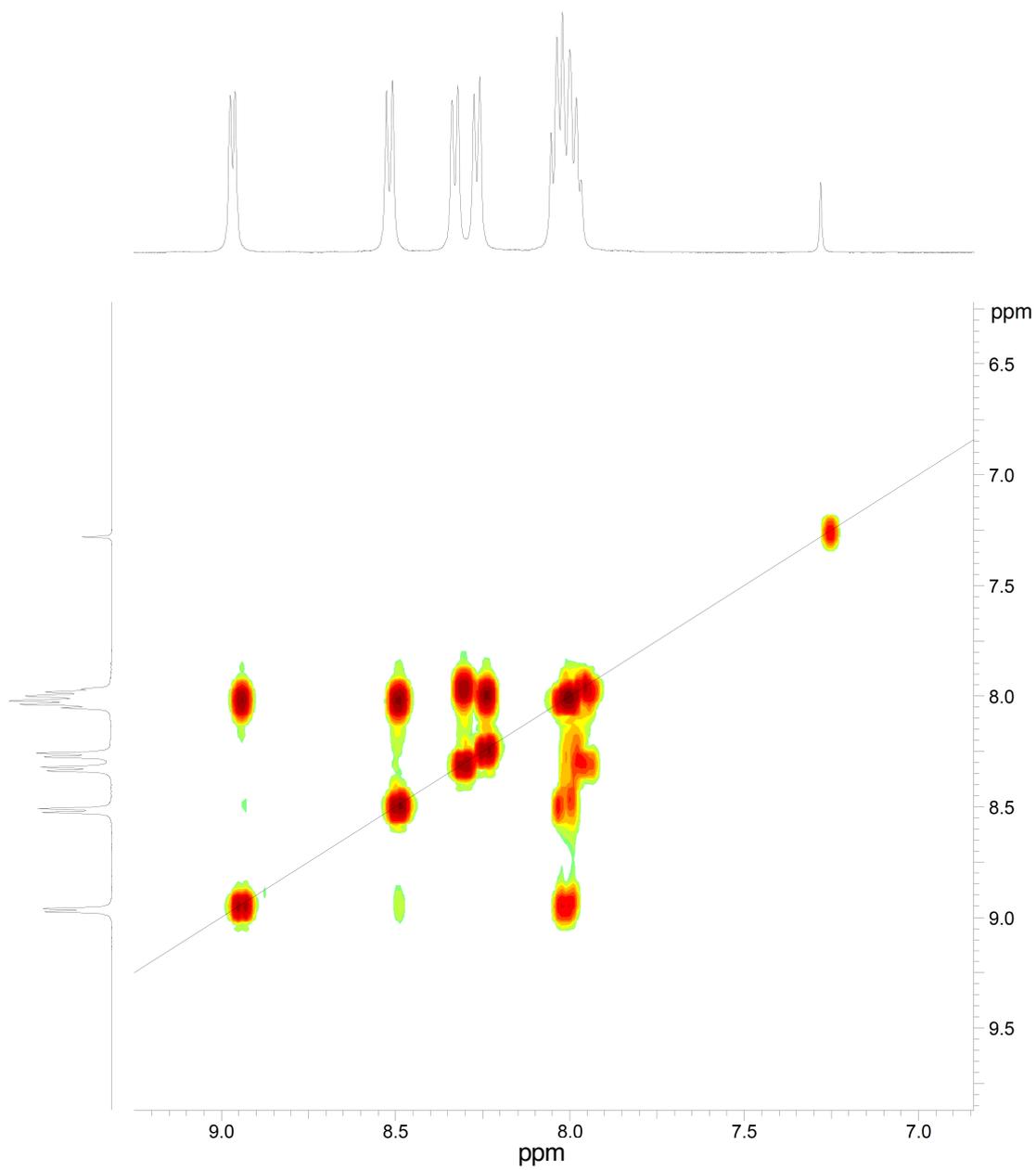
germination rate after 3 weeks (peat moss bed)



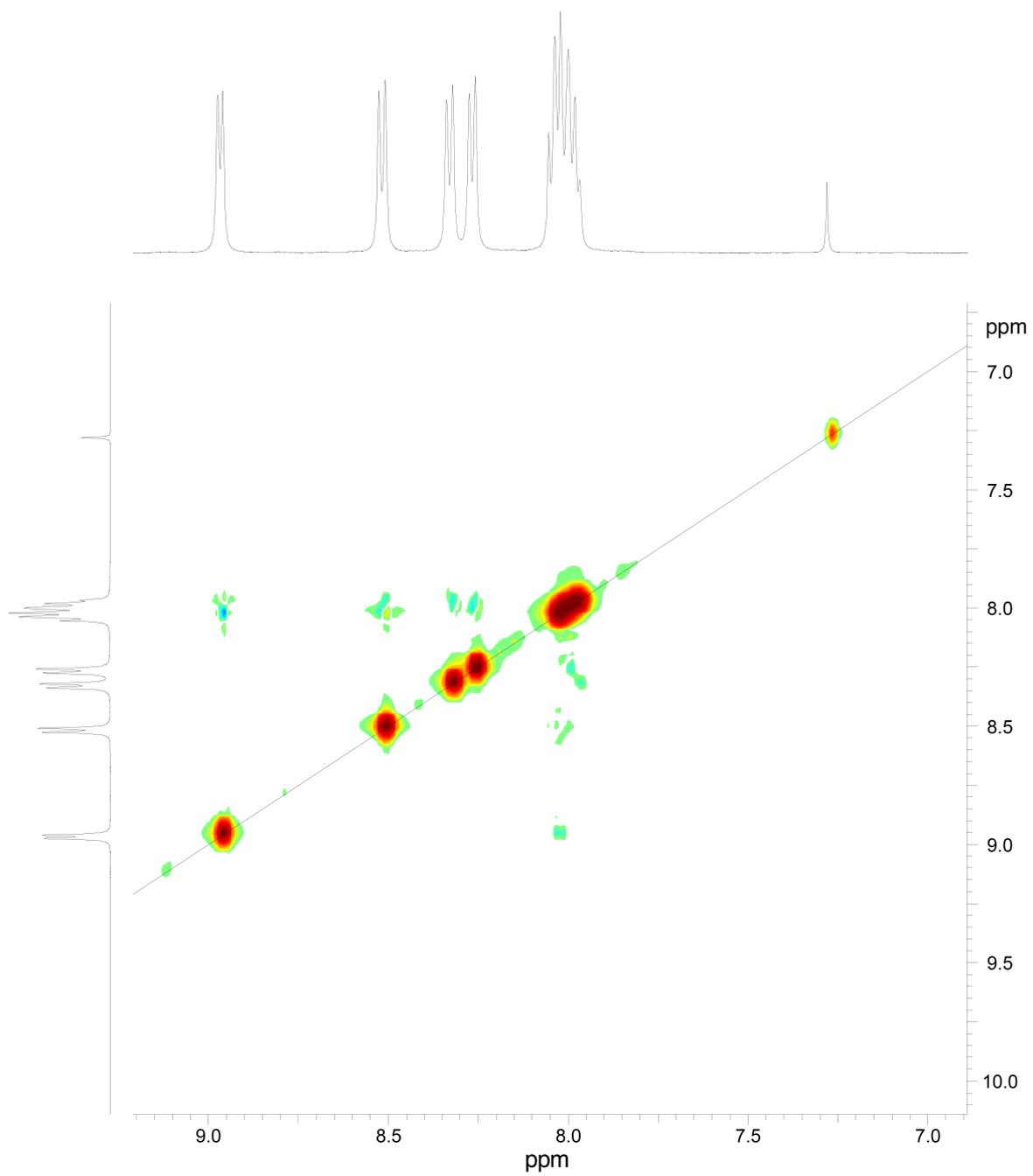
**Figure S3.** Germination rate of *X. complanata* seeds on sterilized peat moss inoculated with *P. rolfsii* strain Y-1 or *P. phenazinium* CK-PC1. Control: non-treated plants. Each value is represented by the mean  $\pm$  SD. Number of replicates were  $n \geq 300$ . Dunnett's test, \*\*\*:  $p < 0.001$ .



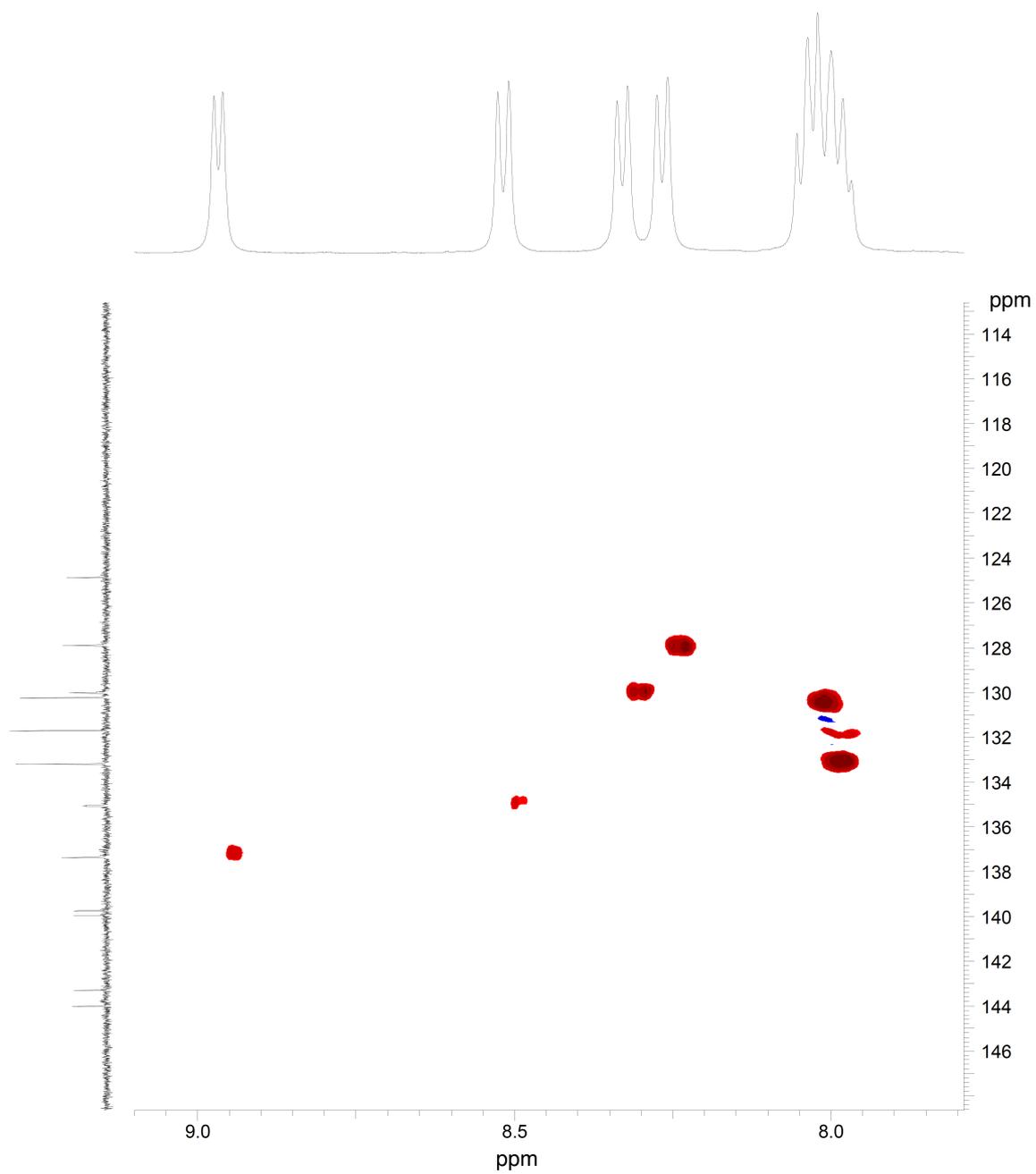
**Figure S4.** NMR spectra of isolated compound **1** from *P. phenazinium* CK-PC1 in CDCl<sub>3</sub>. a) <sup>1</sup>H- and b) <sup>13</sup>C-.



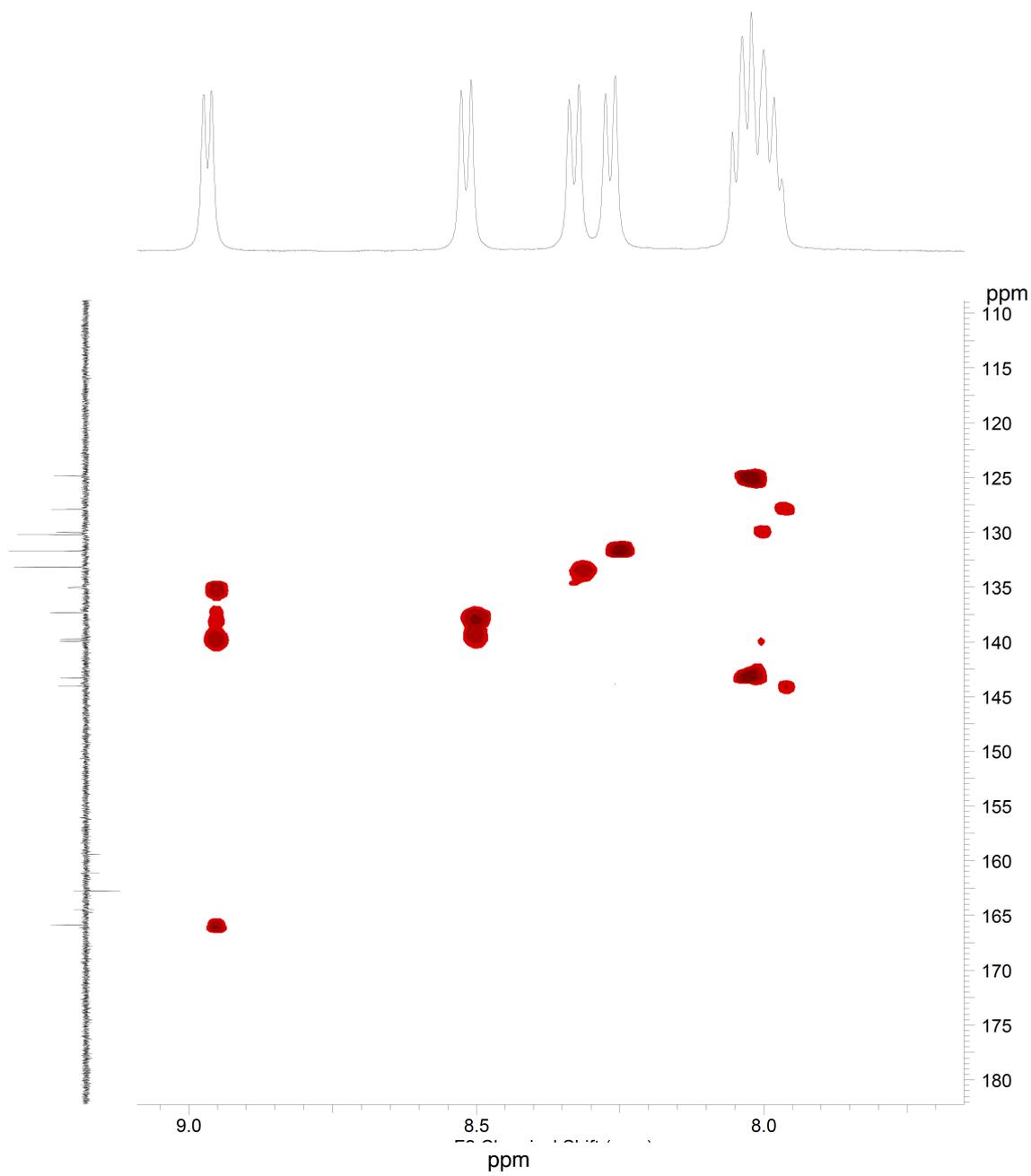
**Figure S5.** COSY spectrum of isolated compound **1** in CDCl<sub>3</sub>



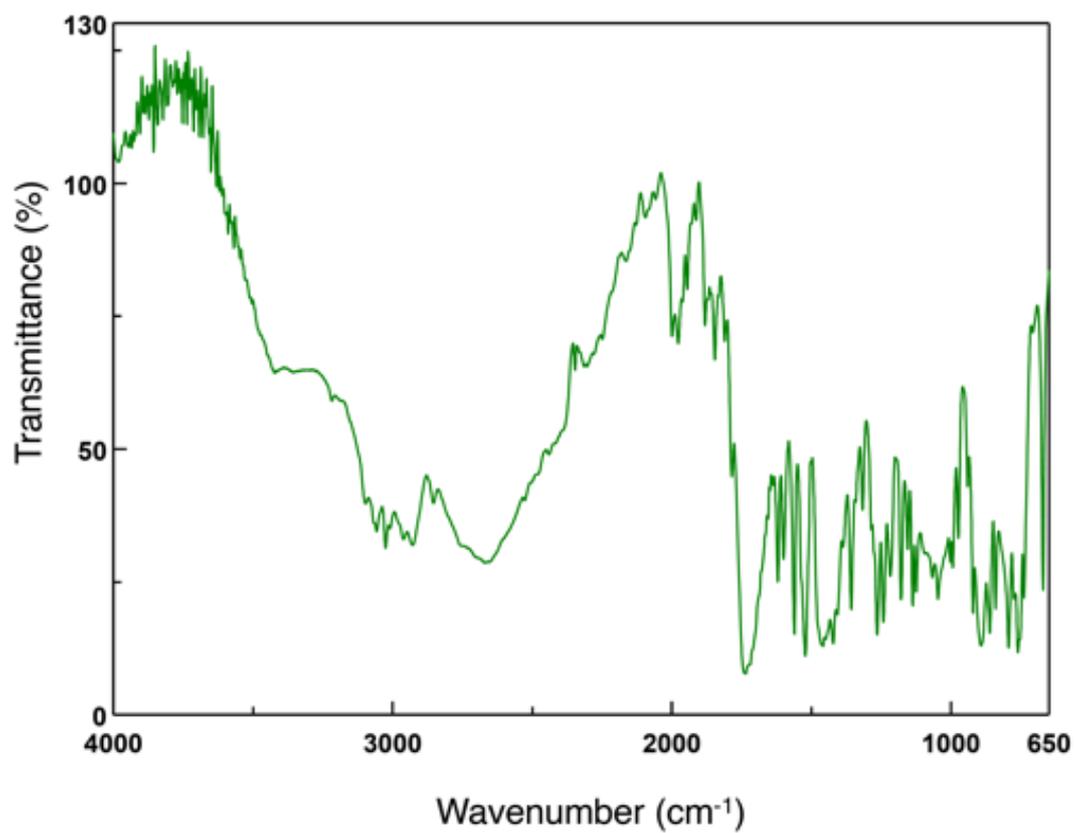
**Figure S6.** NOESY spectrum of isolated compound **1** in CDCl<sub>3</sub>



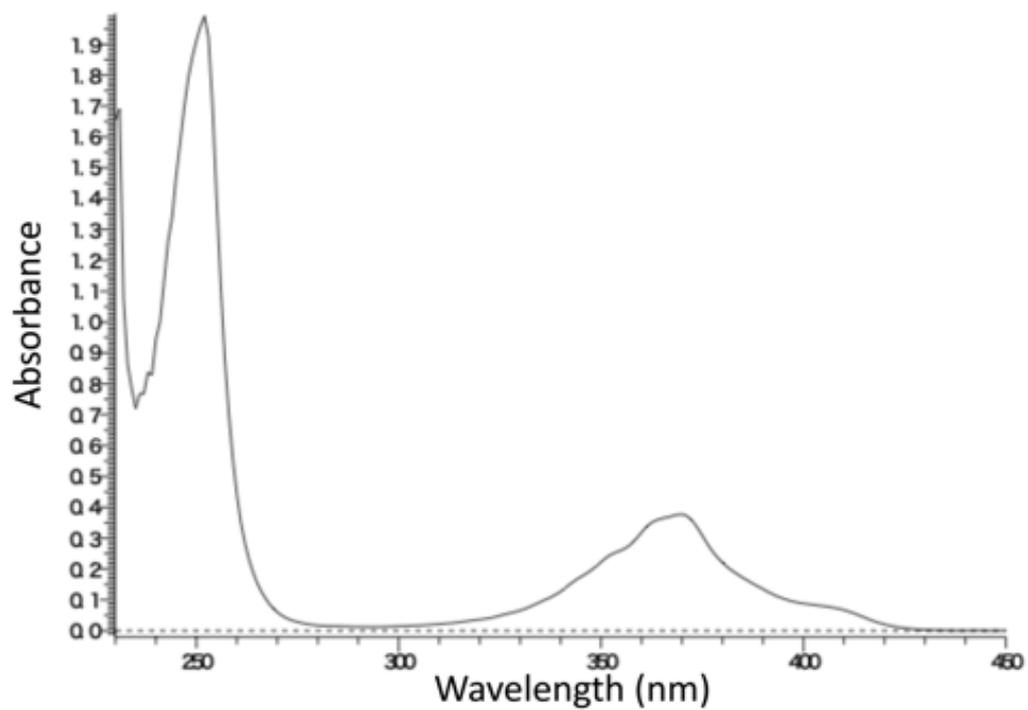
**Figure S7.** HSQC spectrum of isolated compound **1** in CDCl<sub>3</sub>



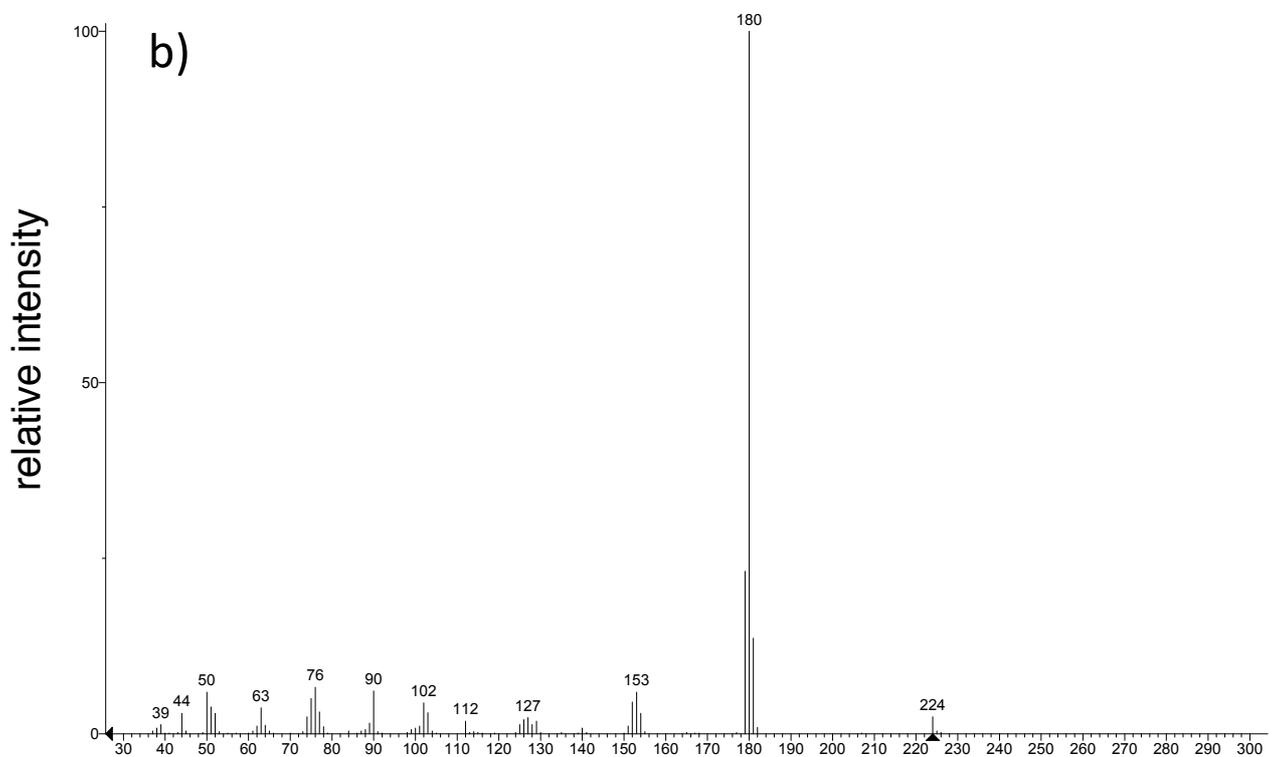
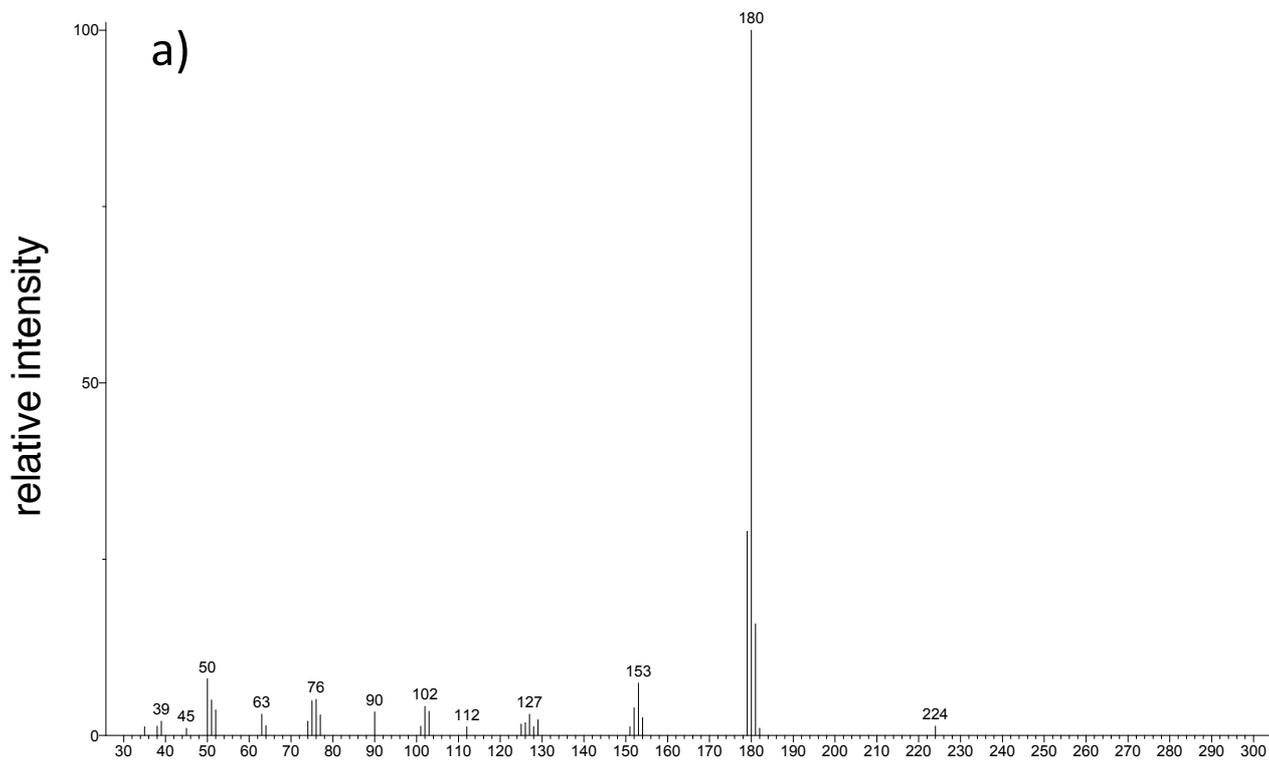
**Figure S8.** HMBC spectrum of isolated compound **1** in CDCl<sub>3</sub>



**Figure S9.** IR spectrum of isolated compound **1**



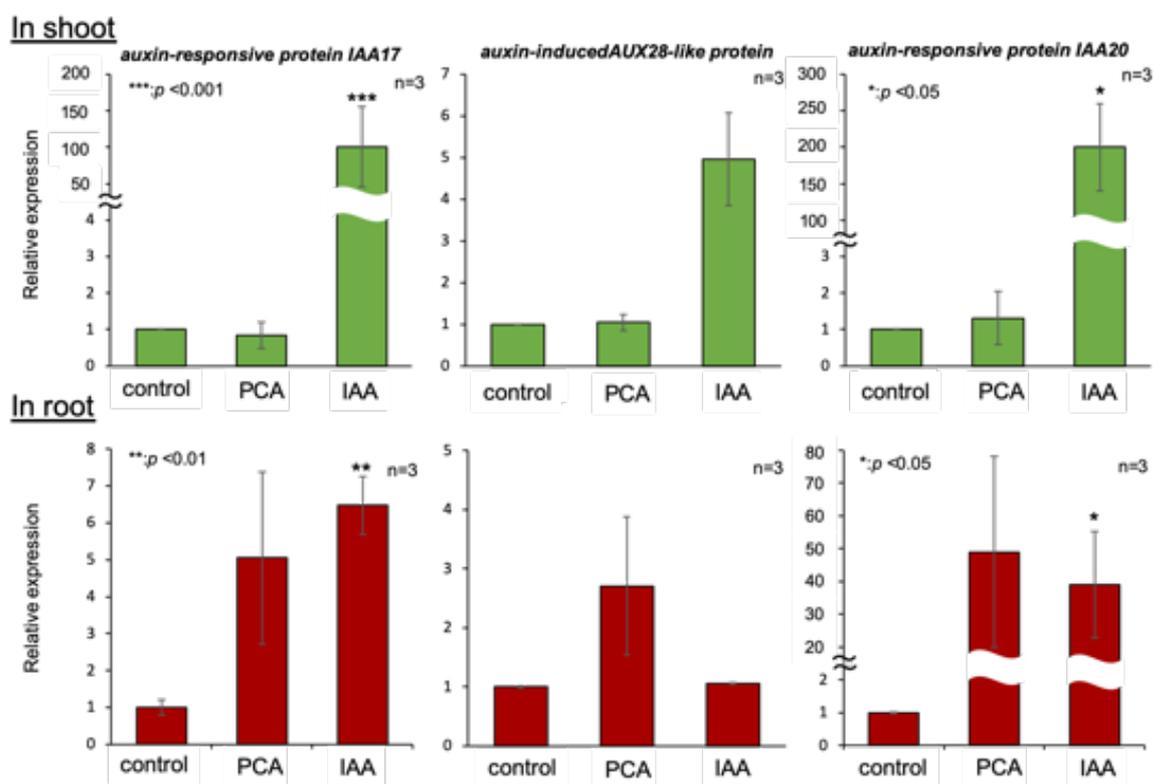
**Figure S10.** UV spectrum of isolated compound **1** in  $\text{CHCl}_3$  (0.0196 mM)  
 $\lambda_{\text{max}}$  ( $\epsilon$ ) 252 (99600), 370 (19000)



**Figure S11.** EI-MS spectrum of compound **1** and authentic compound.

a) EI-MS spectrum of isolated compound **1** from *P. phenazinium* CK-PC-1.

b) EI-MS data of phenazine 1-carboxylic acid obtained from NIST/EPA/NIH Mass Spectral Library.

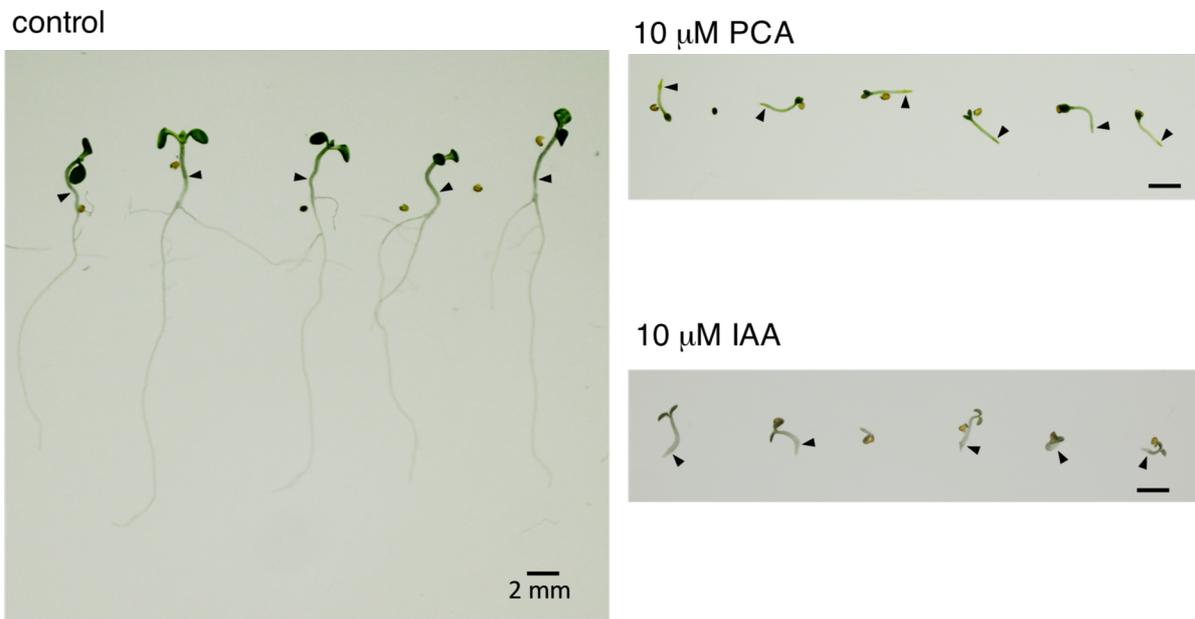


**Figure S12.** Transcription levels of auxin-responding genes in root part by treatment with 10  $\mu$ M PCA (1) solution. Control plant were treated with RO water instead of PCA (1) and IAA solution. Each value is represented by the mean  $\pm$  SD. Dunnett's test, \*:p<0.05, \*\*:p<0.01, \*\*\*:p<0.001.

**Table S1.** Primers designed and used for RT-qPCR for the targeted genes self-checked as original primers.

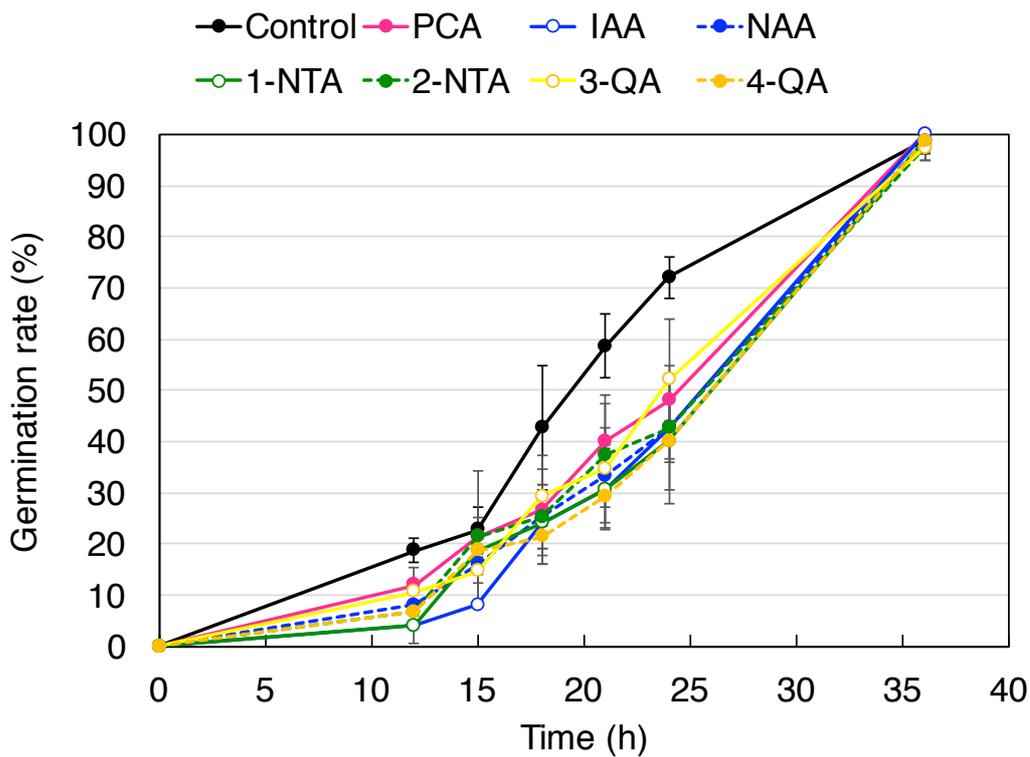
Primer name	Standard curve	correlation coefficient	Amplification efficiency (%)	Primer concentration ( $\mu\text{M}$ )
<i>IAA17</i>	$y=-2.999\ln(x)+31.84$	$R^2=0.922$	115.5	0.20
<i>Aux28</i>	$y=-3.491\ln(x)+25.38$	$R^2=0.972$	110.8	0.15
<i>IAA20</i>	$y=-3.101\ln(x)+32.37$	$R^2=0.947$	110.1	0.20
<i>Skip16</i>	$y=-3.039\ln(x)+31.15$	$R^2=0.946$	113.3	0.15

*Skip16* gene is for house-keeping gene.



**Figure S13.** Effect of PCA on *Arabidopsis thaliana* root elongation.

Control is the *A. thaliana* seeds incubated on 1/2 MS mineral solution-based 1.0% gellan gum plate, whereas the treatments with 10  $\mu$ M IAA or PCA are the gellan gum plates containing 10  $\mu$ M IAA or PCA (1).  $\blacktriangle$  shows the boundary between the hypocotyl and the root.



**Figure S14.** Effect of bi-(or tri-) cyclic carboxylic acids on germination rate of *V. radiata*.

*V. radiata* seeds were cultured in the dark at 25°C for 36 h on 0.3% agar plate (control) and 0.3% agar plate containing 10  $\mu$ M of each bi-(or tri-) cyclic carboxylic acid. Each value is represented by the mean  $\pm$  SD. Number of replicates were  $n = 75$ . Result of Dunnett test, germination rates at 21 and 24 h were significantly lower in all groups treated with carboxylic acids compound than in the control at a 5% significance level.