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

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

Abstract

Symbiosis of *Penicillium rolfsii* Y-1 is essential for the seed germination of Hawaii yellow-eyed grass (*Xyris complanata*). However, the local soil where the plants grow naturally often suppresses the radicle growth of the seedlings. This radicle growth was drastically restored by co-inoculation of *Paraburkholderia phenazinium* isolate CK-PC1, which is a rhizobacterium of *X. complanata.* It was found that the isolate CK-PC1 produced phenazine-1-carboxylic acid (PCA, **1**) as a major metabolite. The biological effects of PCA (**1**) were investigated using the seeds of *X. complanata* and mung bean (*Vigna radiata*), and it was uncovered that the symbiosis of the isolate CK-PC1was essential for the post germination growth of *X. complanata* and the metabolite, PCA (**1**) might partially contribute to promote the growth of the plants.

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

Introduction

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

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

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

Materials and Methods

General

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

Plants, microorganisms and the local soil

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

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

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

Cultivation of X. complanata.

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

Isolation of P. phenazinium CK-PC1.

The strain CK-PC1 was isolated from the rhizosphere of *X. complanata* seedlings, which 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_2SO_4, 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/$CHCl_3$/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. ^{1}H-NMR (270 MHz, $CDCl_3$) 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). ^{13}C-NMR (66.5 MHz, $CDCl_3$) δ: 164.9 (CO),

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 (3), 129.0 (9), 126.9 (6), 123.9 (1). UV ($CHCl_3$) λ_{max} (ε): 252 (99600), 370 (19000). IR ν_{max} cm^{-1}: 3033, 2937, 2657, 175, 1471. HR-FD-MS $[M]^+$ calcd. for $C_{13}H_8N_2O_2$, 224.0586; found, 224.0593. EI-MS *m/z* (rel. int, %), 224.1 $[M]^+$ (0.8), 180.0 (100), 178.9 (24), 152.9 (6.8), 125.9 (2.4), 101.9 (4.1), 76.0 (5.3), 49.8 (6.2).

Epicotyl gravitropism and epicotyl elongation assays using V. radiata seedlings

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

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

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

For the measurement of germination rate, the surface-sterilized seeds of *V. radiata* were placed on a solidified plate (10 ml) using agar (0.3 %) containing 10 μM IAA, PCA (**1**), 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) 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. For the measurements of fresh weight and radicle length, the germinated seeds were treated with IAA, PCA (**1**) and the analogs, respectivlely, and were incubated for 36 h in the same manner as described above.

Statistical analysis.

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

Results

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

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

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

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

To address of the biological effects exhibited by co-inculcation of *P. rolfsii* Y-1 and *P. phenazinium* CK-PC1, the seeds of *X. complanata* were sown on the sterilized local soil. It was found that the radicles of the seedlings treated with *P. rolfsii* Y-1 alone hardly developed (Figure 1c), although better growth of roots and leaves were observed when *P. rolfsii* Y-1 and *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

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

Discussion

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

P. rolfsii Y-1 stimulated seeds germination of *X. complanata*, however, the seedlings subsequently showed poor radicle growth (Figure 1c) and were not able to grow for long periods (Figure 1a). On the other hand, co-inoculation of *P. rolfsii* Y-1 with *P. phenazinium* CK-PC1, which was isolated as a rhizobacterium of *X. complanata*, restored the radicle growth 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 μM 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

339 **References**

340 [1] Cross AT, Turner SR, Renton M, et al. Seed dormancy and persistent sediment seed

341 banks of ephemeral freshwater rock pools in the Australian monsoon tropics. Ann Bot.

342 2015;115:847–859.

343 [2] Finch-Savage WE, Footitt S. Seed dormancy cycling and the regulation of dormancy

344 mechanisms to time germination in variable field environments. J Exp Bot.

345 2017;68:843–856.

[3] Hu XW, Pan J, Min DD, et al. Seed dormancy and soil seedbank of the invasive weed *Chenopodium hybridum* in north-western China. Weed Res. 2017;57:54–64.

[4] Xie X, Yoneyama K, VanAlfen N, et al. The Strigolactone Story. Annu Rev Phytopatho 2010;48:93–117.

[5] Flematti GR, Ghisalberti EL, Dixon KW, et al. A compound from smoke that promotes seed germination. Science. 2004;305:977–977.

[6] Chiwocha SDS, Dixon KW, Flematti GR, et al. Karrikins: A new family of plant growth regulators in smoke. Plant Sci. 2009;177:252–256.

[7] Nelson DC, Riseborough J-A, Flematti GR, et al. Karrikins discovered in smoke trigger *Arabidopsis* seed germination by a mechanism requiring gibberellic acid synthesis and light. Plant Physiol. 2009;149: 863–873.

[8] Arditti J. Factors affecting germination of orchid seeds. Bot Rev. 1967;33:1–97.

[9] Dijk E, Willems JH, Van Andel J. Nutrient responses as a key factor to the ecology of orchid species. Acta Botanica Neerlandica. 1997;46:339–363.

[10] Tamura R, Hashidoko Y, Ogita N, et al. Requirement for particular seed-borne fungi for seed germination and seedling growth of *Xyris complanata*, a pioneer monocot in topsoil-lost tropical peatland in Central Kalimantan, Indonesia. Ecol Res. 2008;23:573–

579.

[11] Kretzer AM, Bruns TD. Use of atp6 in fungal phylogenetics: an example from the boletales. Mol Phylogenet Evol. 1999;13:483–492.

[12] Sugita T, Nishikawa A. Fungal identification method based on DNA sequence analysis: Reassessment of the methods of the pharmaceutical society of Japan and the Japanese pharmacopoeia. J Health Sci. 2003;49:531–533.

[13] Tamura K, Stecher G, Peterson D, et al. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol. 2013;30:2725–2729.

[14] Hashidoko Y, Tada M, Osaki M, et al. Soft gel medium solidified with gellan gum for preliminary screening for root-associating, free-living nitrogen-fixing bacteria inhabiting the rhizoplane of plants. Biosci Biotechnol Biochem. 2002;66:2259–2263.

[15] Lane D. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic Acid Techniques in Bacterial Systematics. Modern Microbiological Methods. New York: John Wiley and Sons; 1991. p. 115–175.

[16] Weisburg W, Barns S, Pelletier D, et al. 16S Ribosomal and amplification for phylogenetic study. J Bacteriol. 1991;173:697–703.

[17] Turner S, Pryer K, Miao V, et al. Investigating deep phylogenetic relationships among

cyanobacteria and plastids by small submit rRNA sequence analysis. J Euk Microbiol. 1999;46:327–338.

[18] Lee KC, Arai T, Ibrahim D, et al. Purification and characterization of a xylanase from the newly isolated *Penicillium rolfsii* c3-2(1) IBRL. Bioresources. 2015;10:1627–1643.

[19] Lee KC, Arai T, Ibrahim D, et al. Purification and characterization of a thermostable laminarinase from *Penicillium rolfsii* c3-2(1) IBRL. Bioresources. 2014;9:1072–1084.

[20] Lee KC, Arai T, Ibrahim D, et al. Characterization of oil-palm trunk residue degradation enzymes derived from the isolated fungus, *Penicillium rolfsii* c3-2(1) IBRL. Environ Technol. 2016;37:1550–1558.

[21] Mehnaz S, Saleem R, Yameen B, et al. Lahorenoic acids A-C, ortho-dialkyl-substituted aromatic acids from the biocontrol strain *Pseudomonas aurantiaca* PB-St2. J Nat Prod. 2013;76:135–141.

[22] Abraham A, Philip S, Jacob MK, et al. Phenazine-1-carboxylic acid mediated anti-oomycete activity of the endophytic *Alcaligenes* sp. EIL-2 against *Phytophthora meadii*. Microbiol Res. 2015;170: 229–234.

[23] Overvoorde P, Fukaki H, Beeckman T. Auxin control of root development. Cold Spring Harb Perspect Biol. 2010;2: a001537.

[24] Wang Y, Wilks J, Danhorn T, et al. Phenazine-1-carboxylic acid promotes bacterial biofilm development via ferrous iron acquisition. J Bacteriol. 2011;193:3606–3617.

[25] Xu S, Pan X, Luo J, et al. Effects of phenazine-1-carboxylic acid on the biology of the plant-pathogenic bacterium *Xanthomonas oryzae* pv. oryzae. Pest Biochem Physiol. 2015;117:39–46.

[26] Reed J. Roles and activities of Aux/IAA proteins in *Arabidopsis*. Trends Plant Sci. 2001;6(9):420–425.

[27] Jain R, Pandey A. A phenazine-1-carboxylic acid producing polyextremophilic *Pseudomonas chlororaphis* (MCC2693) strain, isolated from mountain ecosystem, possesses biocontrol and plant growth promotion abilities. Microbiol Res. 2016;190: 63–71.

[28] Cui QN, Lv HN, Qi ZZ, et al. Cross-regulation between the *phz1* and *phz2* operons maintain a balanced level of fhenazine biosynthesis in *Pseudomonas aeruginosa* PAO1. PLoS One. 2016;11:e0144447.

[29] Peng HS, Zhang PY, Bilal M, et al. Enhanced biosynthesis of phenazine-1-carboxamide by engineered *Pseudomonas chlororaphis* HT66. Microb Cell Factories. 2018;17:117.

[30] Giddens SR, Feng YJ, Mahanty HK. Characterization of a novel phenazine antibiotic

gene cluster in *Erwinia herbicola* Eh1087. Mol Microbiol. 2002;45:769–783.

[31] Mavrodi OV, Mavrodi DV, Parejko JA, et al. Irrigation differentially impacts populations of indigenous antibiotic-producing *Pseudomonas* spp. in the rhizosphere of wheat. Appl Environ Microbiol. 2012;78:3214–3220.

[32] Mavrodi DV, Mavrodi OV, Parejko JA, et al. Accumulation of the antibiotic phenazine-1-carboxylic acid in the rhizosphere of dryland cereals. Appl Environ Microbiol. 2012;78:804–812.

[33] Schneemann I, Wiese J, Kunz A, et al. Genetic approach for the fast discovery of phenazine producing bacteria. Mar Drugs. 2011;9:772–789.

[34] Upadhyay A, Srivastava S. Phenazine-1-carboxylic acid is a more important contributor to biocontrol *Fusarium oxysporum* than pyrrolnitrin in *Pseudomonas fluorescens* strain Psd. Microbiol. Res. 2011;166:323-335.

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 μM.

	Epicotyl gravitropism (°) (n =15)	Epicotyl length (mm) (n =30)	Germination rate (%) (n =100×3)	Fresh weight (mg) (n =75)	Radicle length (mm) (n =15)
Control[a)]	15 ± 3	6 ± 1	62.6 ± 8.3	236.8 ± 5.3	26.3 ± 0.8
PCA	29 ± 3*	11 ± 1***	37.3 ± 4.6**	201.3 ± 4.3***	17.4 ± 0.6***
IAA	34 ± 2***	12 ± 1***	21.3 ± 2.3***	202.1 ± 4.2***	13.8 ± 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

Figure captions

Figure 1. Restoration of *X. complanata* growth by co-inoculation with a rhizobacterium, *P. phenazinium* CK-PC1 from non-sterilized local soil.

The seedlings of *X. complanata* were cultivated for 5 weeks on sterilized local soil. (a) Seedlings of *X. complanata* treated with germination stimulating fungus *P. rolfsii* Y-1. (b) Seedlings of *X. complanata* co-inoculated with germination stimulating fungus *P. rolfsii* Y-1 and the suspension of non-sterilized local soil. (c) Roots of *X. complanata* seedlings with *P. rolfsii* Y-1. (d) Roots of *X. complanata* seedlings with *P. rolfsii* Y-1 + *P. phenazinium* CK-PC1. Arrows indicate radicles. (e) Growth promotion of *X. complanata* seedlings by *P. rolfsii* Y-1 and *P. phenazinium* CK-PC1. Seedlings germinated without microbial inoculation were used as control. The leaf length of the seedlings cultivated for five weeks were measured. Each value is represented by the mean ± SD. Number of replicates were shown in the Figure 1e. Dunnett's test, ***:$p < 0.001$.

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

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 μmol 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 ± 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 μM of each bi-(or tri-) cyclic carboxylic acid. Each value is

464 represented by the mean ± 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

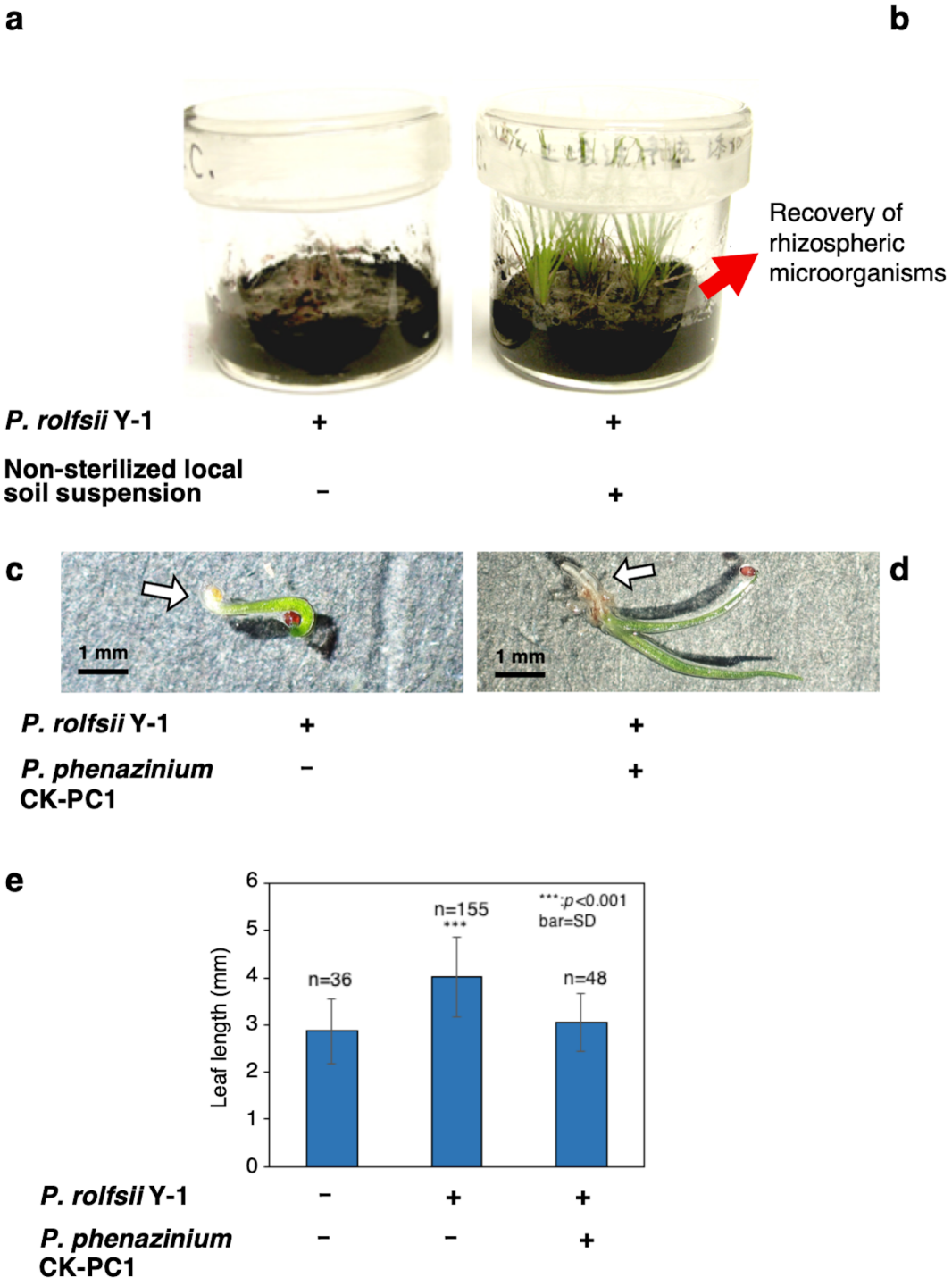
a
b
Recovery of rhizospheric microorganisms
P. rolfsii Y-1 + +
Non-sterilized local soil suspension − +
c
d
1 mm
1 mm
P. rolfsii Y-1 + +
P. phenazinium CK-PC1 − +
e
Leaf length (mm)
0
1
2
3
4
5
6
n=36
n=155
***
n=48
***:p<0.001
bar=SD
P. rolfsii Y-1 − + +
P. phenazinium CK-PC1 − − +

Figure 1

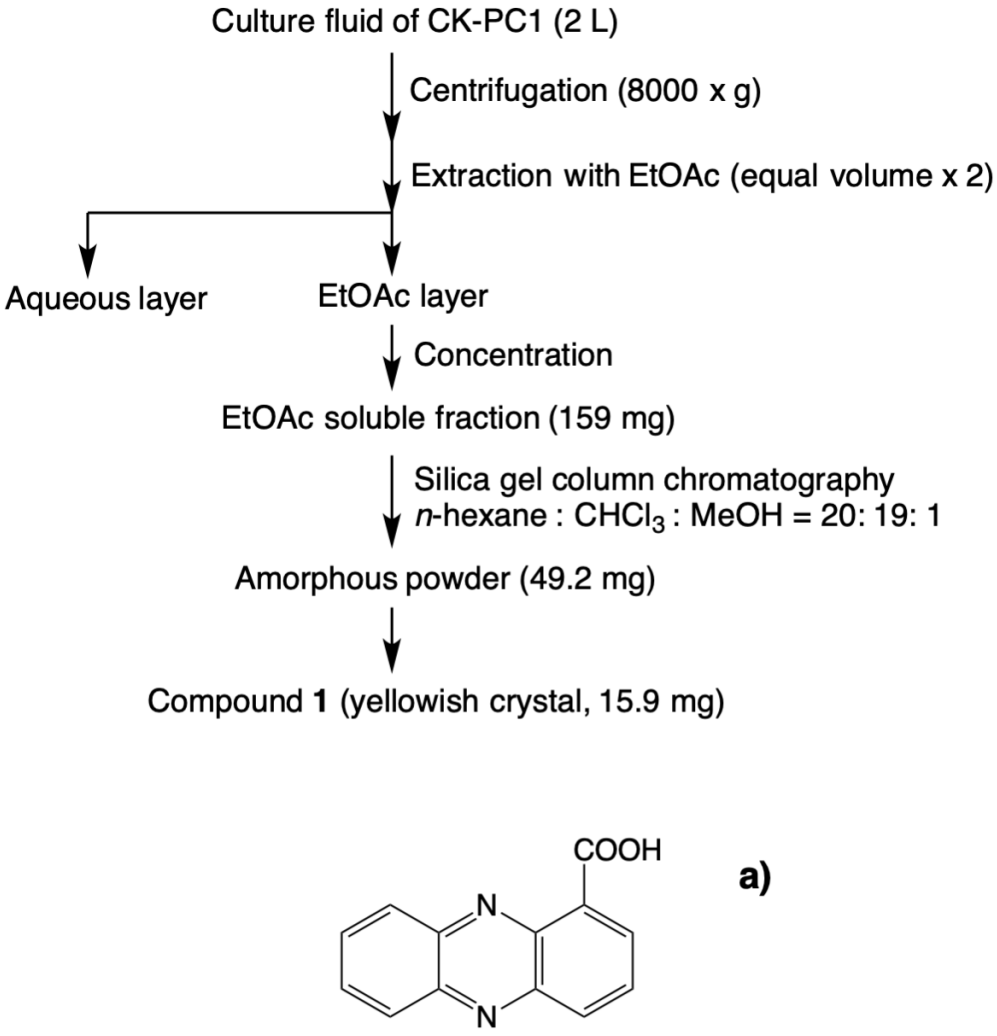
Culture fluid of CK-PC1 (2 L)
Centrifugation (8000 x g)
Extraction with EtOAc (equal volume x 2)
Aqueous layer
EtOAc layer
Concentration
EtOAc soluble fraction (159 mg)
Silica gel column chromatography
n-hexane : CHCl3 : MeOH = 20: 19: 1
Amorphous powder (49.2 mg)
Compound 1 (yellowish crystal, 15.9 mg)
COOH
N
N
a)

Figure 2

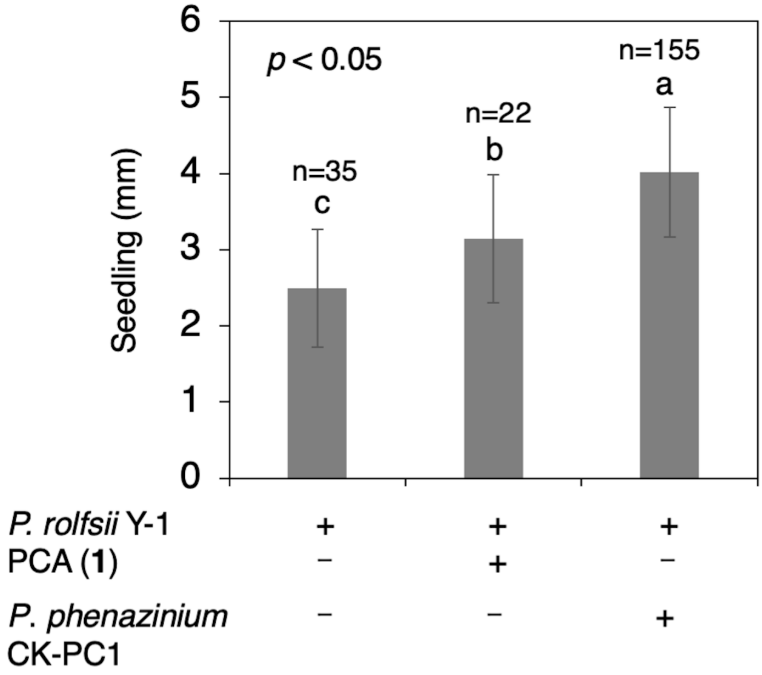
6
5
4
3
2
1
0
Seedling (mm)
p < 0.05
n=35
c
n=22
b
n=155
a
P. rolfsii Y-1
PCA (1)
P. phenazinium
CK-PC1
+
−
−
+
+
−
+

Figure 3

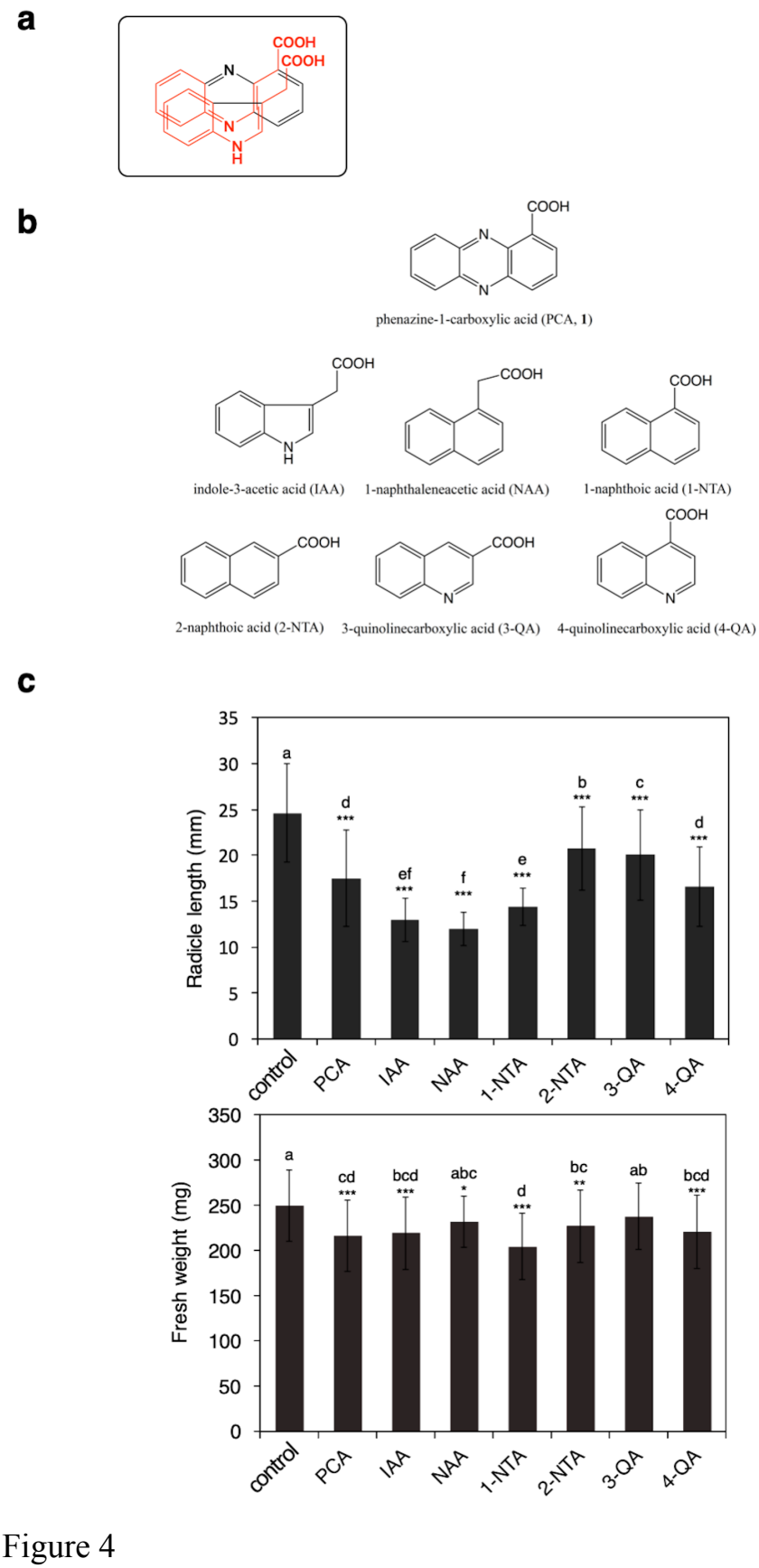
a
COOH
COOH
N
N
N
H
b
COOH
N
N
phenazine-1-carboxylic acid (PCA, 1)
COOH
N
H
indole-3-acetic acid (IAA)
COOH
1-naphthaleneacetic acid (NAA)
COOH
1-naphthoic acid (1-NTA)
COOH
2-naphthoic acid (2-NTA)
COOH
N
3-quinolinecarboxylic acid (3-QA)
COOH
N
4-quinolinecarboxylic acid (4-QA)
c
Radicle length (mm)
35
30
25
20
15
10
5
0
a
d
***
ef
***
f
***
e
***
b
***
c
***
d
***
control
PCA
IAA
NAA
1-NTA
2-NTA
3-QA
4-QA
Fresh weight (mg)
350
300
250
200
150
100
50
0
a
cd
***
bcd
***
abc
*
d
***
bc
**
ab
bcd
***
control
PCA
IAA
NAA
1-NTA
2-NTA
3-QA
4-QA

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 rolfsii* 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 μM 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 (1) 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_2 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 μL of 3 M sodium acetate and 125 μL of 95% EtOH and allowed to stand overnight at -30°C. Next, the solution was centrifuged at 14,000 rpm at room temperature, and the supernatant-removed precipitates were washed once with 625 μL 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 μL RNase-free water. The 0.5 μg 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°C for 15 min, 85°C for 5 s, and cooled to 8°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′, *Tm* 60.9/58.7 °C), *AUX28-2f/AUX28-2r* (5′-ATG TTC AGC TCC TTC ACC-3′/5′-ATC CAG TCA CCA TCC TTG TC-3′, *Tm* 58.3/62.0 °C), and *IAA20-1f/ IAA20-1r* (5′-GTG AAG GTC TAC ATG GAA GG-3′/5′-ATT CAT CTC TGT CCC CCA-3′, *Tm* 59.1/61.0°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′, *Tm* 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 μL 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 μL 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 μM 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.

References

[1] Yamamoto K, Mori H, Imaseki H. Novel messenger-RNA sequences induced by indole-3-acetic-acid in sections of elongating hypocotyls of mung bean (*Vigna radiate*). Plant Cell Physiol. 1992;33:13–20.

[2] Borges A, Tsai SM, Caldas DGG. Validation of reference genes for RT-qPCR normalization in common bean during biotic and abiotic stresses. Plant Cell Rep. 2012;31:827–838.

[3] Wasano N, Sugano M, Nishikawa K, et al. Root-specific induction of early auxin-responsive genes in *Arabidopsis thaliana* by cis-cinnamic acid. Plant Biotechnol. 2013;30:465–471.

[4] Steenackers W, Klima P, Quareshy M, et al. cis-Cinnamic acid is a novel, natural auxin efflux inhibitor that promotes lateral root formation. Plant Physiol. 2017;173:552–565.

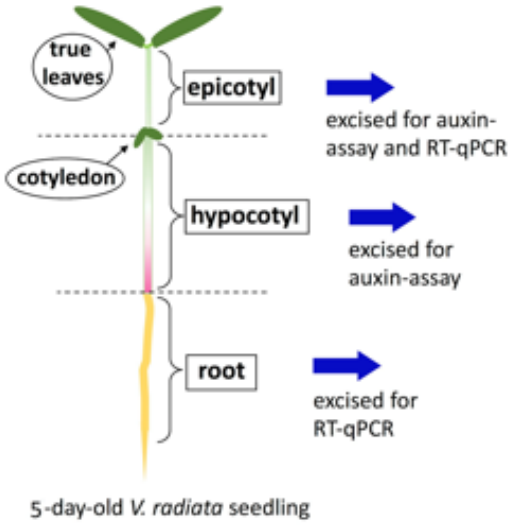

Figure S1. Names of each part in *V. radiata* seedlings.

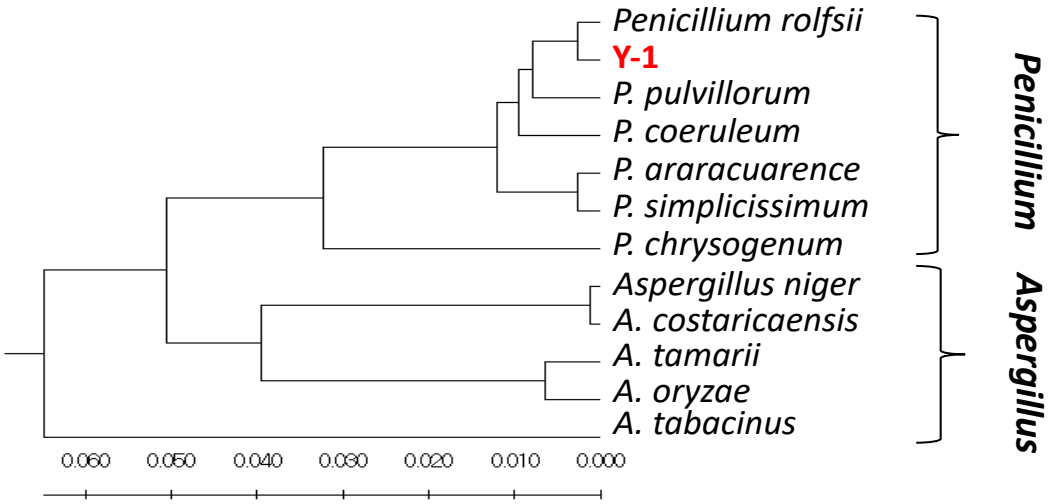

Figure S2. Phylogenetic analysis of *Penicillium* sp. Y-1 strain using ITS1-ITS4 region. The phylogenetic relationship of *Penicillium* sp. Y-1 strain was computed, along with 11 reference species of *Penicillium* and *Aspergillus* fungi selected from several groups of the fungi imperfecti. This neighbor-joining 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.

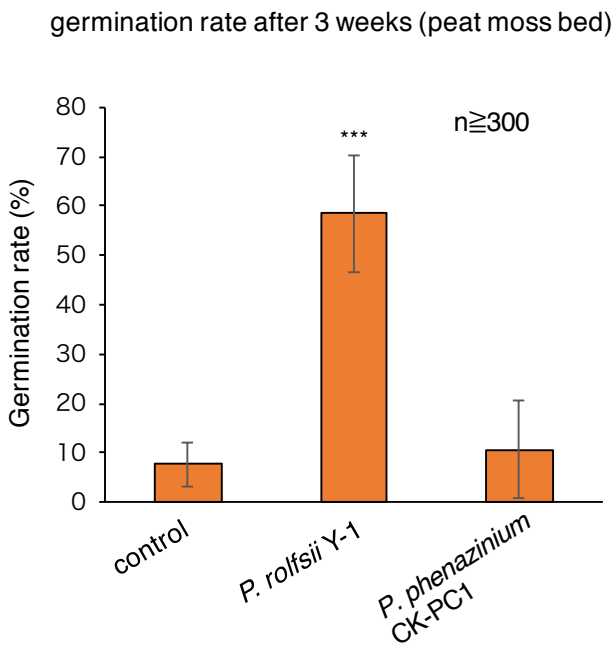

Figure S3. Germination rate of *X. complanata* seeds on sterilized peat moss inoculated with *P. rolfsii strain* Y-1 or *P. phenazinium* CK-PC1. Control: non-treated plants. Each value is represented by the mean ± SD. Number of replicates were n≧300.
Dunnett's test, ***:$p < 0.001$.

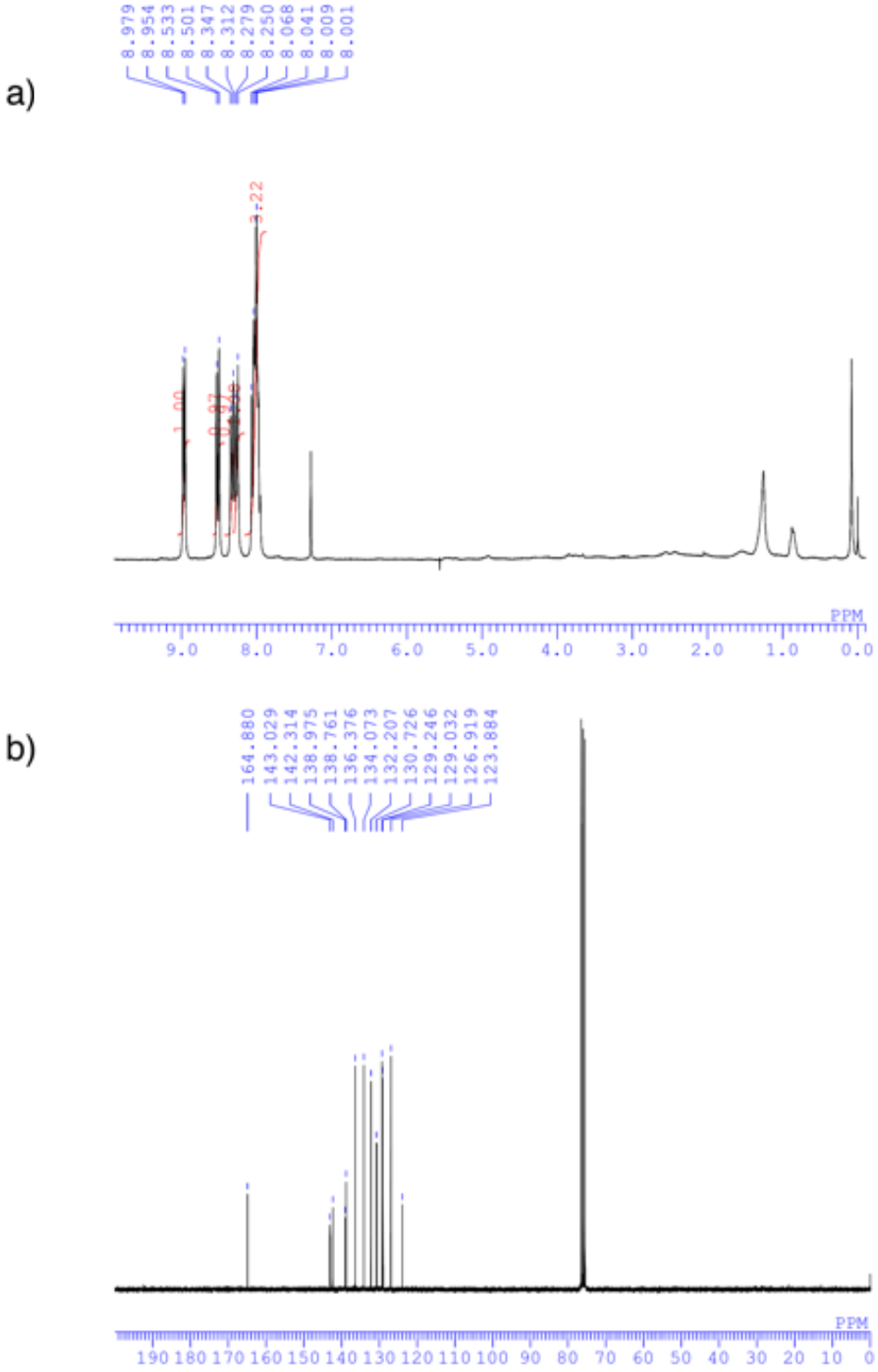

Figure S4. NMR spectra of isolated compound **1** from *P. phenazinium* CK-PC1 in $CDCl_3$. a) ^{1}H- and b) ^{13}C-.

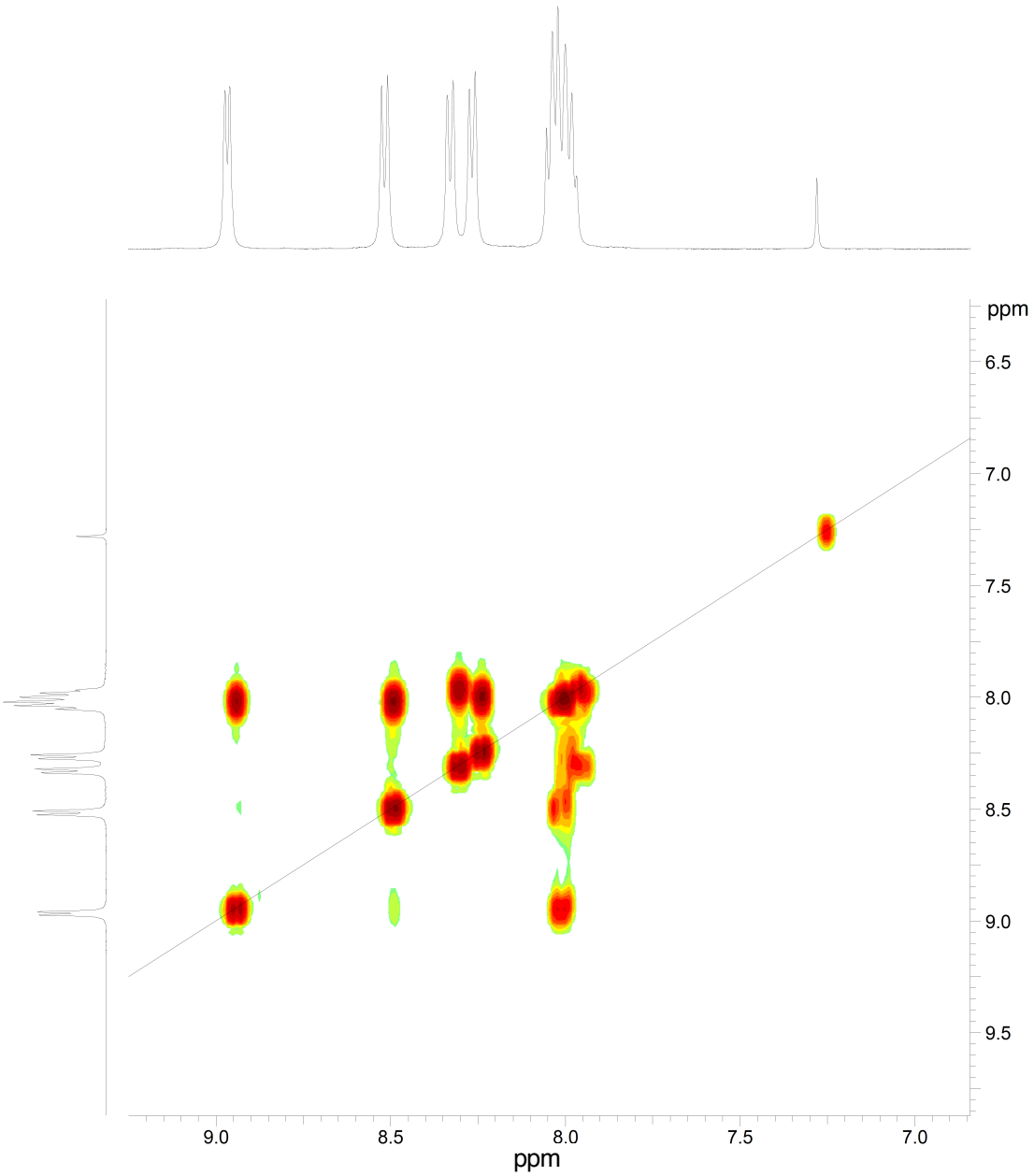

Figure S5. COSY spectrum of isolated compound **1** in $CDCl_3$

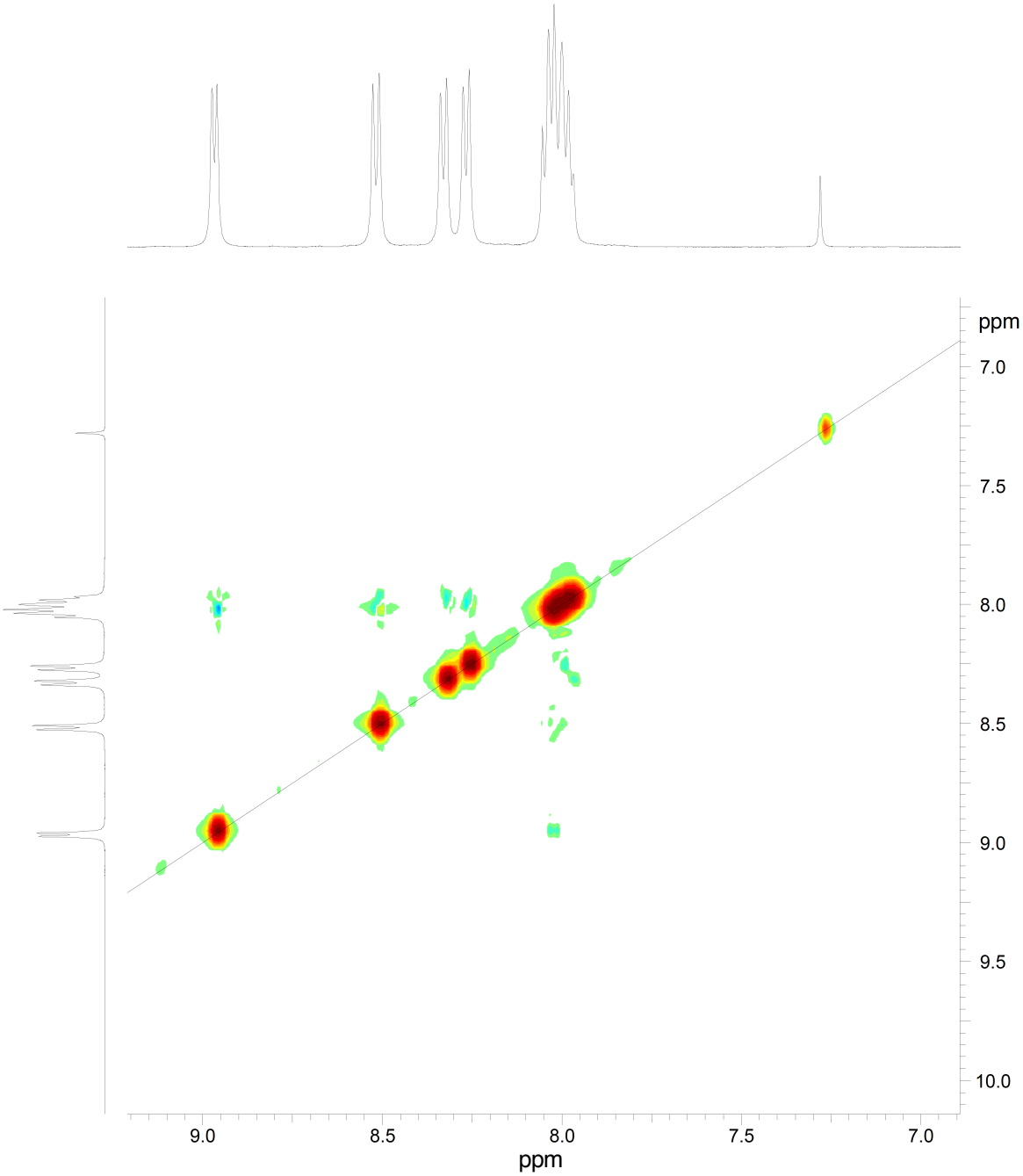

Figure S6. NOESY spectrum of isolated compound **1** in $CDCl_3$

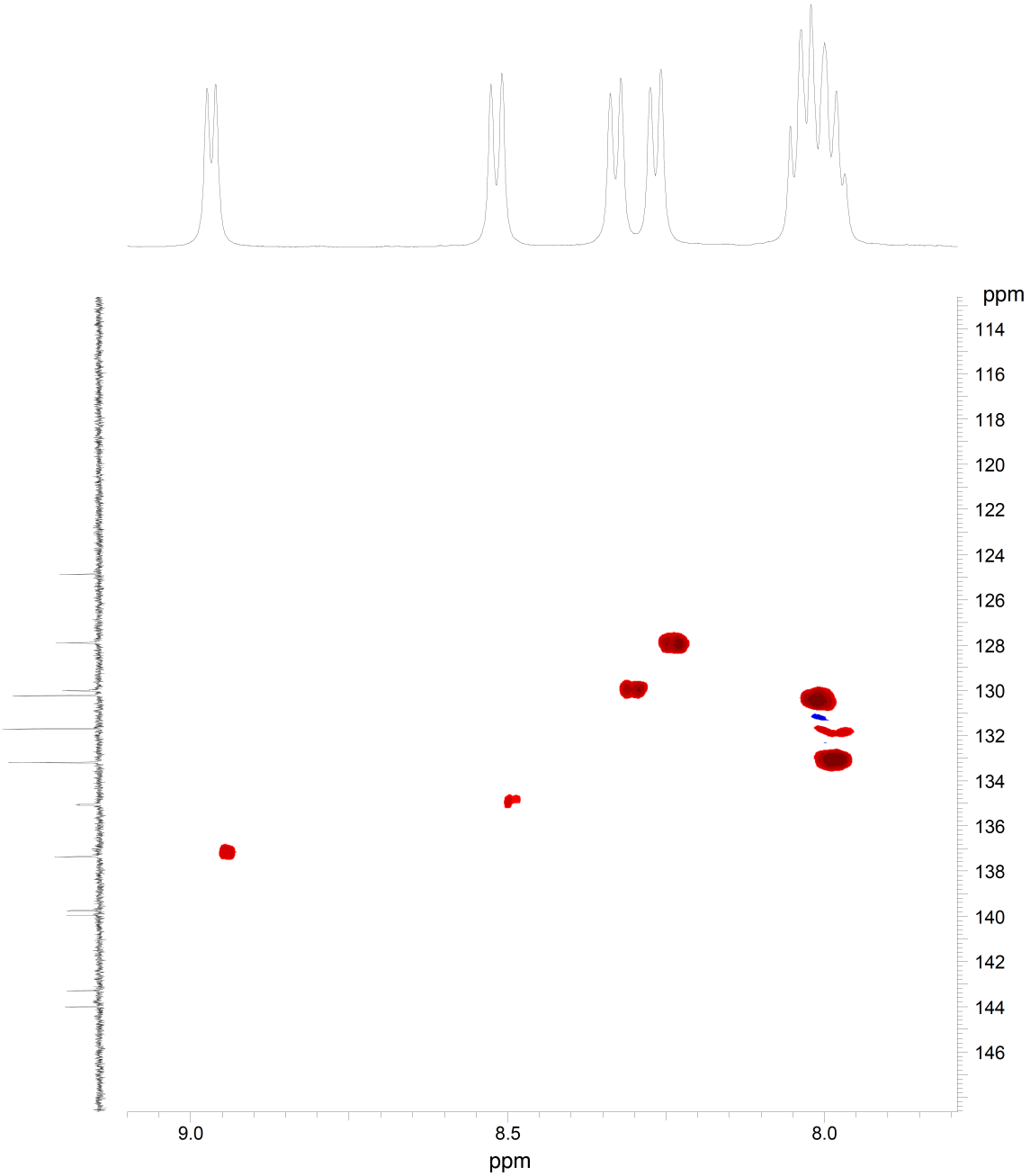

Figure S7. HSQC spectrum of isolated compound **1** in $CDCl_3$

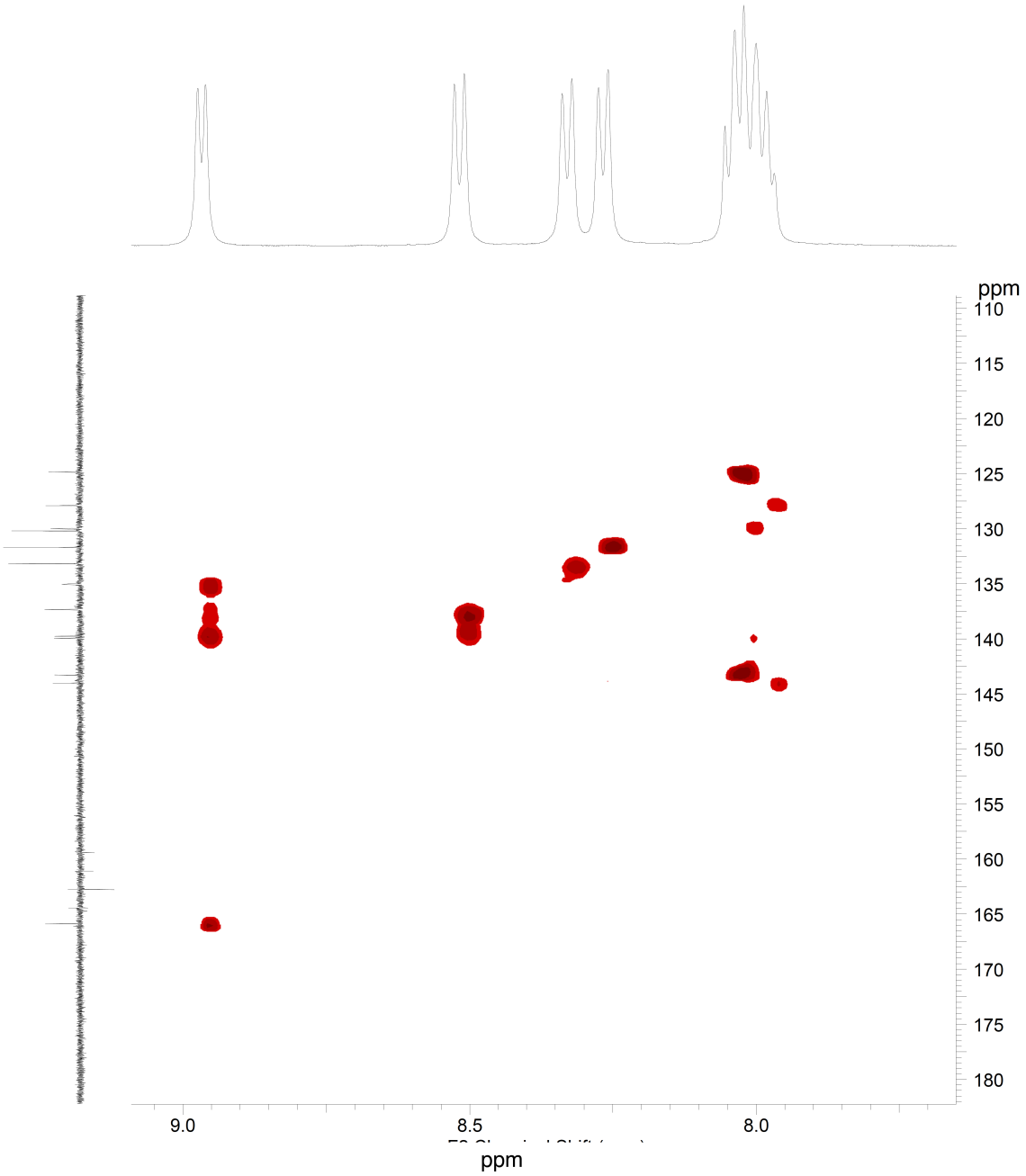

Figure S8. HMBC spectrum of isolated compound **1** in $CDCl_3$

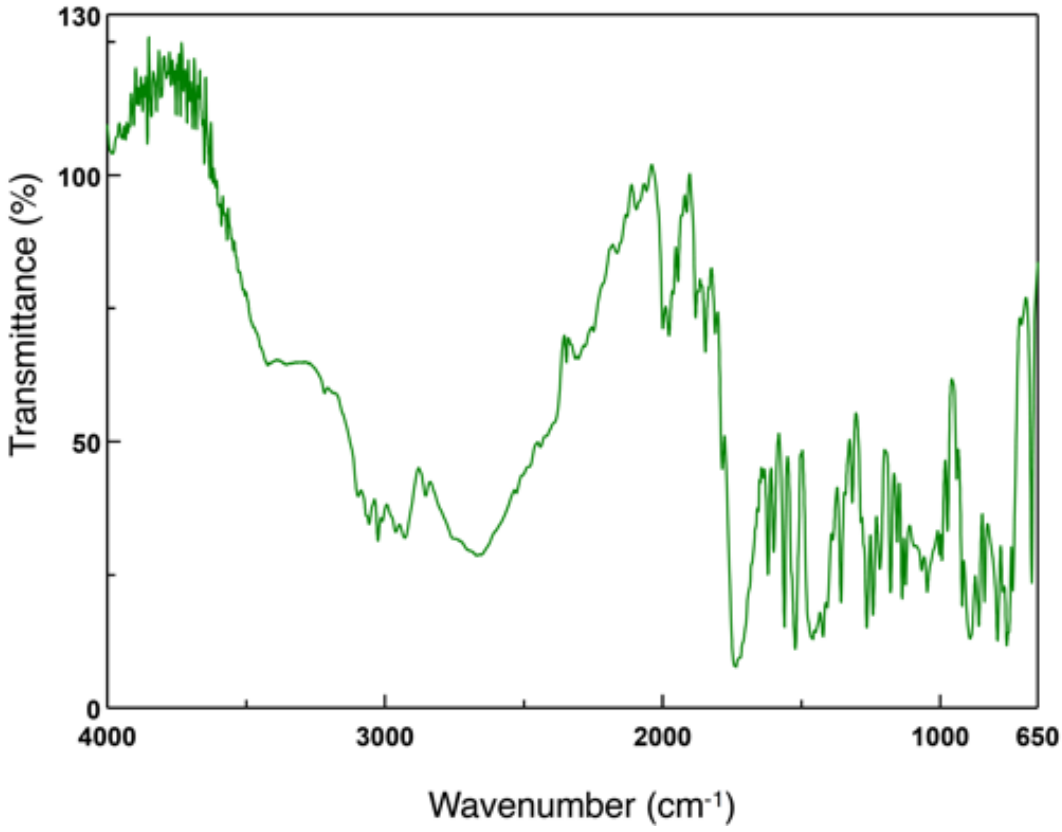

Figure S9. IR spectrum of isolated compound **1**

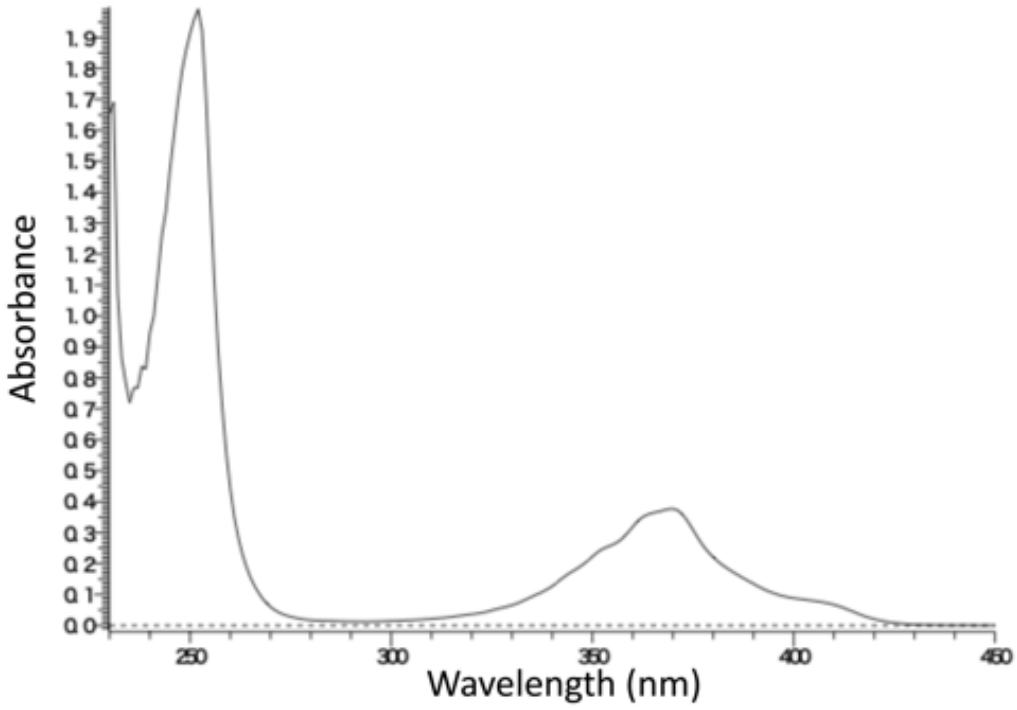

Figure S10. UV spectrum of isolated compound **1** in $CHCl_3$ (0.0196 mM)
λmax (ε) 252 (99600), 370 (19000)

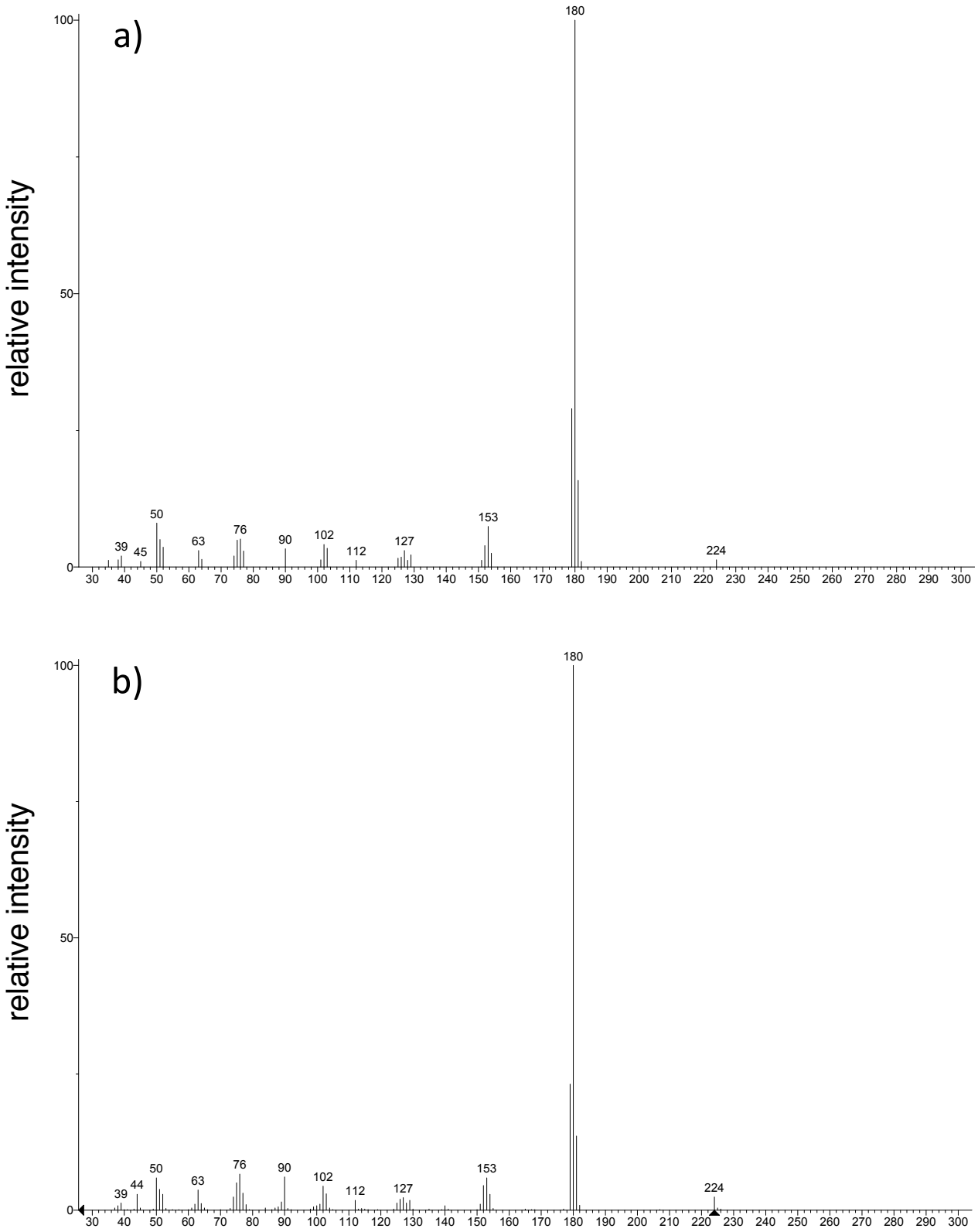

Figure S11. EI-MS spectrum of compound **1** and authentic compound.
a) EI-MS spectrum of isolated compound **1** from *P. phenazinium* CK-PC-1.
b) EI-MS data of phenazine 1-carboxylic acid obtained from NIST/EPA/NIH Mass Spectral Library.

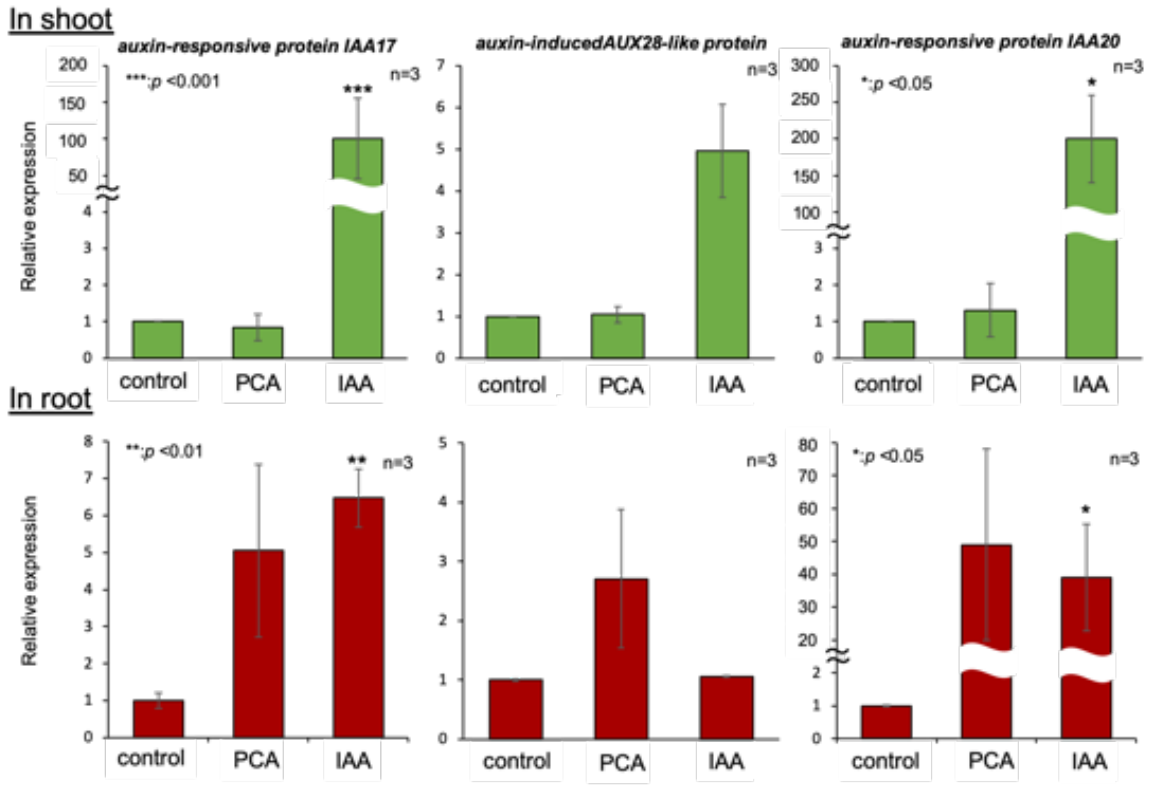

Figure S12. Transcription levels of auxin-responding genes in root part by treatment with 10 μM PCA (**1**) solution.
Control plant were treated with RO water instead of PCA (**1**) and IAA solution. Each value is represented by the mean ± SD. Dunnett's test, *:p<0.05, **:p<0.01, ***:p<0.001.

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 (μM)
IAA17	y=-2.999ln(x)+31.84	R^2=0.922	115.5	0.20
Aux28	y=-3.491ln(x)+25.38	R^2=0.972	110.8	0.15
IAA20	y=-3.101ln(x)+32.37	R^2=0.947	110.1	0.20
Skip16	y=-3.039ln(x)+31.15	R^2=0.946	113.3	0.15

Skip16 gene is for house-keeping gene.

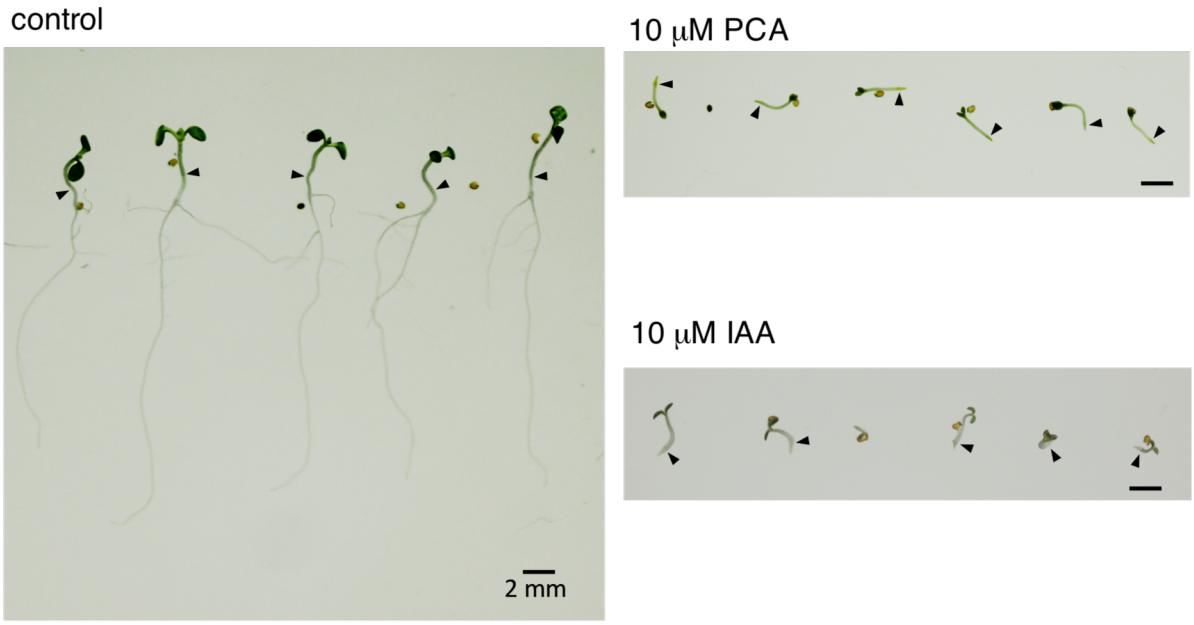

Figure S13. Effect of PCA on *Arabidopsis thaliana* root elongation.
Control is the *A. thalinana* seeds incubated on 1/2 MS mineral solution-based 1.0% gellan gum plate, whereas the treatments with 10 μM IAA or PCA are the gellan gum plates containing 10 μM IAA or PCA (**1**) . ▲shows the boundary between the hypocotyl and the root.

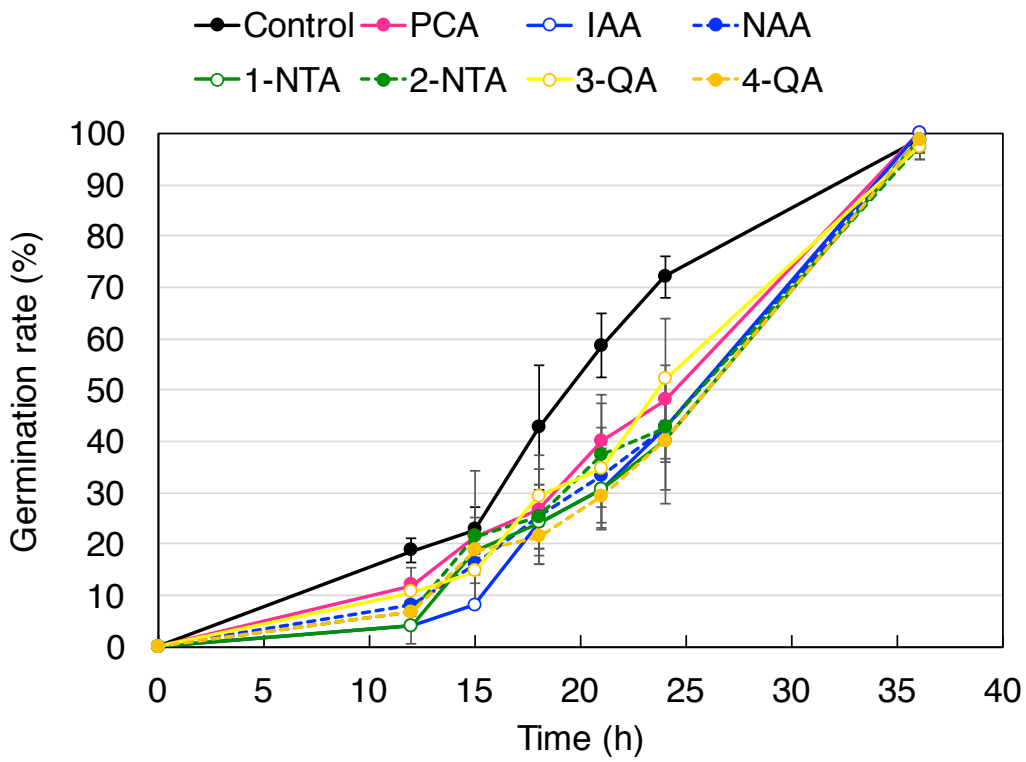

Figure S14. Effect of bi-(or tri-) cyclic carboxylic acids on germination rate of *V. radiata.*

V. radiata seeds were cultured in the dark at 25℃ for 36 h on 0.3% agar plate (control) and 0.3% agar plate containing 10 μM of each bi-(or tri-) cyclic carboxylic acid. Each value is represented by the mean ± 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.