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# Insights on the susceptibility of plant pathogenic fungi to phenazine-1-carboxylic acid and its chemical derivatives

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*Pseudomonas chlororaphis* subsp. *aureofaciens* strain M71 produced two phenazine compounds as main secondary metabolites. These metabolites were identified as phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH P). In this study, the spectrum of the activity of PCA and 2-OH P was evaluated against a group of crop and forestal plant pathogenic fungi by an agar plate bioassay. PCA was active against most of the tested plant pathogens, while 2-OH P slightly inhibited a few fungal species. Furthermore, four semisynthesised derivatives of PCA (phenazine-1-carboxymethyl, phenazine-1-carboxamide, phenazine-1-hydroxymethyl and phenazine-1-acetoxymethyl) were assayed for their antifungal activity against 11 phytopathogenic species. Results showed that the carboxyl group is a structural feature important for the antifungal activity of PCA. Since the activity of phenazine-1-carboxymethyl and phenazine-1-carboxamide, the two more lipophilic and reversible PCA derivatives remained substantially unaltered compared with PCA.

**Keywords:** *Pseudomonas chlororaphis* subsp. *aureofaciens*; phenazine derivatives; antifungal activity



## 1. Introduction

Phenazines are a large group of nitrogen-containing heterocyclic compounds produced by members of *Pseudomonas, Streptomyces* and a few other bacterial genera from soil or marine habitats (Abken et al., 1998; Laursen & Nielsen, 2004). These compounds showed an interesting biological activity that prompted the development of synthetic analogues with antimicrobial, antitumor, antimalaria and antiparasitic activities. More than 100 phenazines of natural origin and 6000 synthetic phenazines have been reported during the last century (Laursen & Nielsen, 2004; L.S. Pierson & E.A. Pierson, 2010). Natural phenazines are generally simple functionalised phenazine derivatives. They include terpenoid phenazines, phenazines derived from both saphenic and griseoluteic acids and others. Synthetic phenazines include phenazine-1-carboxamide, dimeric phenazines,

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phenazine-6,11-dione, phenazine-5,10-dioxide, benzophenazine-5-sulfonic acid and analogues (Cimmino et al., 2012)

The genus *Pseudomonas* contains a number of species that share traits that can be exploited for plant protection. They produce different kinds of antimicrobial compounds with a wide spectrum of activity, such as the phenazines (Weller, 2007). In particular, members of the species *P. aeruginosa, P. fluorescens* and *P. chlororaphis* are able to produce these phenazine antibiotic compounds that subsequently represent the main secondary metabolites responsible for the biological control of important plant pathogens, namely *Gaeumannomyces graminis* var. *tritici, Fusarium oxysporum* f.sp. *radicis-lycopersici* and *Rhizoctonia solani* (Chin-A-Woeng et al., 1998; Liu et al., 2007; Shanmugaiah, Mathivanan, & Varghese, 2010; Thomashow, Weller, Bonsall, & Pierson, 1990).

Recently, Raio et al. (2011) reported that *P. chlororaphis* subsp. *aureofaciens* strain M71 showed to be a potential agent for the control of *Seiridium cardinale,* the causative agent of cypress (*Cupressus sempervirens* L.) bark canker disease. Production of phenazine-1-carboxylic acid (PCA, 1, Figure 1) by strain M71 involved the control of this plant pathogen. This compound was able to inhibit the *in vitro* growth of *S. cardinale* and other cypress pathogenic fungi, such as *Diplodia cupressi, Seiridium cupressi* and *Seiridium unicorne,* while the 2-hydroxyphenazine (2-OH P, **2**, Figure 1) the second phenazine isolated from the culture filtrates of strain M71 lacked this activity (Raio et al., 2011).

Although several works have been documented so far on the importance of the phenazines in the biological control of phytopathogenic fungi, little is known on how their activity can vary against different fungal species. PCA extracted from *P. fluorescens* strain 2-79 was screened against several fungi and bacteria and revealed the greatest inhibitory activity against *G. graminis* var. *tritici* and *Pythium* spp. (Gurusiddaiah, Weller, Sarkar, & Cook, 1986). Subsequently, Mazzola, Fujimoto, Thomashow, and Cook (1995) evidenced that some *G. graminis* var. *tritici* isolates were able to tolerate PCA at a concentration of $1\,\mu g\,mL^{-1}$, but so far nothing is known on the effect of PCA on other plant pathogenic fungi, especially on fungal species that attack forestry plants.

Therefore, the first aim of this work was to assess the susceptibility of a group of fungi pathogenic to crop and forestal plants towards PCA and 2-OH P produced by *P. chlororaphis* subsp. *aureofaciens* strain M71. A further aim was to define the structural features involved in the antifungal activity of PCA, assayed in comparison with four semisynthetic derivatives.

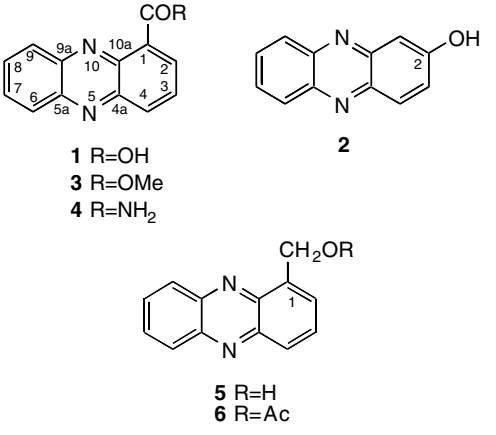


Figure 1. Structures of PCA (**1**) and its derivatives (**3–6**) and that of 2-OH P (**2**).

## 2. Results and discussion

PCA and 2-OH P (respectively, **1** and **2** in Figure 1) were evaluated for their antifungal activity against several plant pathogenic fungi at concentrations of 10, 25 and 50 $\mu g\,mL^{-1}$ (Table 1). PCA was the most effective in the inhibition of the major part of the plant pathogenic fungi evaluated in this work (Table 1). Moreover, these results are in total agreement with previously published data that reported phenazine-producing *Pseudomonas* species as biocontrol agents of plant pathogenic fungi (Pierson & Thomashow, 1992; Raio et al., 2011; Vincent et al., 1991). On the contrary, 2-OH P did not affect the growth of 15 out of the 25 tested fungal species. Four fungi were slightly inhibited (less than 10% reduction in colony radial growth), while the colony development of four *Fusarium* species and of *G. graminis* var. *tritici* was reduced between 18% and 34% at a concentration of 50 $\mu g\,mL^{-1}$ (Table 1).

These data lead us to hypothesise that the production of 2-OH P in strain M71 could be related to some other biological mechanisms. Recently, Maddula, E.A. Pierson, and L.S. Pierson (2008) showed that some mutant derivatives of *P. chlororaphis* strain 30-84, that produced only PCA or overproduced 2-hydroxy-phenazine-1-carboxylic acid (2-OH PCA), were altered in their ability to form biofilms and inhibit *G. graminis* var. *tritici*. Thus, these evidences encourage further investigations on the role played by 2-OH P in the ecology of strain M71.

Each fungal species showed a different sensitivity to PCA, even though increasing concentration of the compound in the medium was always associated with a strong reduction of colony growth, with the only exception of *R. solani*. PCA was able to totally inhibit the growth of *Phytophthora nicotianae* and *G. graminis* var. *tritici* at concentrations of 25 and 50 $\mu g\,mL^{-1}$. Moreover, *P. cinnamomi* and *Pythium ultimum* grew on PDA plates amended with PCA 25 $\mu g\,mL^{-1}$ while *P. nicotianae* and *P. cambivora* were strongly inhibited at this concentration (Table 1). *P. cinnamomi, P. ultimum* and *R. solani* overgrew on the PDA plates amended with 25 $\mu g\,mL^{-1}$ PCA, but it was observed that their mycelia were thinner than the group (data not shown). In a follow-up experiment carried out in liquid media, each of these three fungal species exhibited a slower growth rate than the group when grown in a tube containing PDB amended with 25 $\mu g\,mL^{-1}$ of PCA (Figure 2). At the end of this second experiment, organic extracts of the broth media were analysed by TLC for the presence of PCA degradation products. For the three fungal species mentioned above, no PCA degradation products were detected. The absence of degradation products leads us to hypothesise that *P. cinnamomi, P. ultimum* and *R. solani* share some biological mechanism that allows them to tolerate PCA. One of these mechanisms might be represented by ATP-binding cassette (ABC) transporters. The activity of BcatrB (Stefanato et al., 2009) in *Botrytis cinerea* provides protection against PCA and phenazine-1-carboxyamide when these compounds were present with the fungus on tomato leaves at the same time (Schoonbeek, Raaijmakers, & De Waard, 2002).

These results and those reported by Raio et al. (2011) strongly suggest that PCA is involved in the antagonistic activity of strain M71. Since fungi tested in this work showed a variable sensitivity to this antibiotic compound (Table 1 and Figure 2), a study on the relationship between the structure and antifungal activity of PCA was carried out to further investigate the chemical features of PCA associated with its antifungal activity. This study was carried out through the preparation of semisynthetic derivatives from PCA.

PCA (**1**) was converted into four derivatives by chemical modification of the carboxyl group. In fact, by the reaction with diazomethane, **1** was quantitatively converted into the corresponding methyl ester (**3**, Figure 1), a reversible derivative (Axel, 1983), which, in

Table 1. Susceptibility of plant pathogenic fungi and oomycetes towards PCA and 2-OH P[a].

| Phytopathogenic fungi and oomycetes | PCA | | | 2-OH P | | |
|---|---|---|---|---|---|---|
| | 10 μg mL$^{-1}$ | 25 μg mL$^{-1}$ | 50 μg mL$^{-1}$ | 10 μg mL$^{-1}$ | 25 μg mL$^{-1}$ | 50 μg mL$^{-1}$ |
| *Alternaria alternata* | 3c[b] | 30b | 64a | 3c | 3c | 3c |
| *Botrytis cinerea* | n.d.[c] | 41b | 63a | n.d. | 0c | 0c |
| *Ceratocystis platani* | n.d. | 12b | 34a | n.d. | 0c | 0c |
| *Chryphonectria parasitica* | n.d. | 1b | 23a | n.d. | 6c | 12b |
| *Colletotrichum gloesporoides* | 0c | 36b | 66a | 0c | 0c | 0c |
| *Fusarium oxysporum* f.sp. *basilici* | 0c | 42b | 62a | 0c | 0c | 0c |
| *Fusarium oxysporum* f.sp. *lycopersici* | 0e | 40b | 74a | 8d | 12cd | 18c |
| *Fusarium oxysporum* f.sp. *radicis-lycopersici* | 52c | 82b | 98a | 31d | 29d | 34d |
| *Fusarium sambucinum* | 22c | 41b | 56a | 25c | 28c | 27c |
| *Fusarium semitectum* | 0e | 58b | 81a | 1e | 7d | 22c |
| *Fusarium solani* | 0e | 41b | 58a | 0e | 5d | 9c |
| *Gaeumannomyces graminis* var. *tritici* | 91b | 100a | 100a | 5d | 8cd | 23c |
| *Ophiostoma novo-ulmi* | n.d. | 53b | 76a | n.d. | 0c | 0c |
| *Phaeomoniella chlamidospora* | n.d. | 18b | 25a | n.d. | 0c | 0c |
| *Phaeoacremonium aleophylum* | n.d. | 18a | 15a | n.d. | 9b | 11b |
| *Phytophthora cactorum* | n.d. | 0c | 12b | n.d. | 13b | 18a |
| *Phytophthora cambivora* | n.d. | 63b | 82a | n.d. | 0c | 0c |
| *Phytophthora cinnamomi* | n.d. | 5b | 13a | n.d. | 0c | 0c |
| *Phytophthora nicotianae* | 77b | 100a | 100a | 5c | 6c | 6c |
| *Pyrenochaeta lycopersici* | 0c | 29b | 63a | 0c | 0c | 0c |
| *Pythium ultimum* | 0b | 0b | 100a | 0b | 0b | 0b |
| *Rhizoctonia solani* | 0a | 0a | 0a | 0a | 0a | 0a |
| *Seiridium cardinale* | n.d. | 29b | 38a | n.d. | 0c | 0c |
| *Sclerotinia sclerotiorum* | 0c | 31b | 77a | 0c | 0c | 0c |
| *Thielaviopsis basicola* | 0c | 31b | 43a | 3c | 5c | 3c |

Notes: Treatments with the same letters are not statistically different according to Tukey's test ($p < 0.01$).
[a]The values reported in the table represent the percentage of the reduction of radial mycelial growth when fungal strains and oomycetes were growing on PDA plates amended with PCA or 2-OH P compared with the not amended PDA.
[b]The reduction of radial mycelial growth expressed as a percentage.
[c]n.d. = not determined.

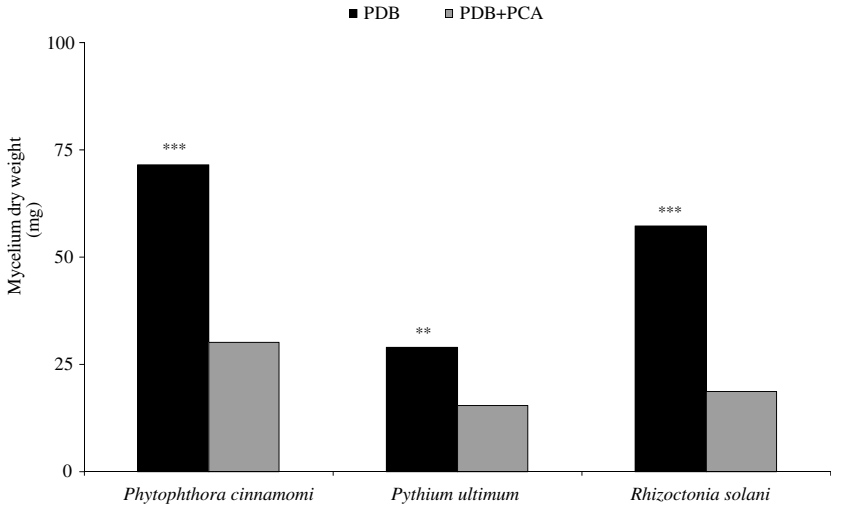


Figure 2. Effects of PCA at 25 μg mL$^{-1}$ on the mycelium dry weight of three phytopathogenic microorganisms.
Notes: Differences between means were compared using Student's *t* test. *** $p < 0.001$ ** $p < 0.1$.



turn was converted into the corresponding amide (**4**, Figure 1), another reversible derivative, by nucleophilic acyl substitution using ammonia (Chin-A-Woeng et al., 1998). Furthermore, the $LiAlH_4$ reduction of the methyl ester (**3**) yielded 1-hydromethylphenazine (**5**, Figure 1) (L. Birkhofer & A. Birkhofer, 1952; Ichiro & Kan, 1964), which showed a permanent modification of the carboxyl group. This latter derivative was converted into the corresponding acetyl derivative (**6**, Figure 1) (Ichiro & Kan, 1964), in which the primary hydroxy group was reversibly modified. The **3**–**6** derivative structures confirmed by spectroscopic investigation and some data, essentially $^1$H NMR and ESI, are reported here for the first time.

A group of 11 microorganisms including tolerant isolates (i.e. *R. solani*), highly sensitive (i.e. *G. graminis* var. *tritici*) and slightly sensitive to PCA (*A. alternata*) at 25 μg mL$^{-1}$ were selected among the 25 species considered previously in order to evaluate the antifungal activity of the derivatives **3–6** compared with the parent PCA (Table 2). PCA proved to be the most effective compound against *B. cinerea, F. oxysporum* f.sp. *radicis-lycopersici*, *G. graminis* var. *tritici*, *Ophiostoma novo-ulmi*, *P. cambivora* and *S. cardinale*. At the same time, *P. ultimum*, was not affected by any derivatives while *A. alternata* was inhibited by all derivatives at a higher percentage than PCA.

A remarkable reduction or the total loss of antifungal activity was observed for nine isolates in the case of the hydroxymethyl and its corresponding acetyl derivative (**5** and **6**). This effect was probably due to the reduction of the carboxyl group to a primary hydroxy group (Table 2), outlining that the carboxyl group is associated with the inhibitory activity of PCA against different fungal species. This is also supported by the comparison of PCA and 2-OH P activities.

Interestingly, corresponding reversible methyl ester and amide derivatives (**3** and **4**) showed an antifungal activity similar to PCA when tested against *G. graminis* var. *tritici*. Probably, derivatives **3** and **4** were enzymatically hydrolysed into PCA by the fungus according to a well-known mechanism that is frequently called 'lethal metabolism' (Kamrin, 1990). In comparison with PCA, a remarkable reduction in the inhibitory

Table 2. Susceptibility of 11 phytopathogenic fungi and oomycetes to PCA (**1**) and its derivatives (**3–6**).

| Phytopathogenic fungal strains and oomycetes | Compounds | | | | |
|---|---|---|---|---|---|
| | **1**[a] | **3** | **4** | **5** | **6** |
| *Alternaria alternata* | 30d[b] | 53a | 35d | 47b | 38c |
| *Botrytis cinerea* | 41a | 18c | 30b | 10d | 14cd |
| *Fusarium oxysporum* f.sp. *radicis-lycopersici* | 82a | 37e | 69b | 57c | 43d |
| *Gaeumannomyces graminis* var. *tritici* | 100a | 98ab | 92b | 74c | 37d |
| *Ophiostoma novo-ulmi* | 53b | 100a | 70ab | 20c | 21c |
| *Phytophthora cambivora* | 63ab | 49b | 77a | 21c | 14c |
| *Phytophthora cinnamomi* | 5c | 5c | 29a | 0c | 0c |
| *Pythium ultimum* | 0a | 0a | 0a | 0a | 0a |
| *Rhizoctonia solani* | 0c | 36a | 26b | 0c | 26b |
| *Sclerotinia sclerotiorum* | 31a | 0c | 13b | 0c | 0c |
| *Seiridium cardinale* | 29b | 32b | 52a | 33b | 27b |

Notes: Treatments with the same letters are not statistically different according to Tukey's test ($p < 0.01$)

[a]**1**: phenazine-1-carboxylic acid; **3**: phenazine-1-carboxymethyl; **4**: phenazine-1-carboxamide; **5**: phenazine-1-hydroxymethyl; **6**: phenazine-1-acetoxymethyl.

[b]The reduction of radial mycelial growth expressed as a percentage.



activity was recorded for derivatives **3** and **4** against *F. oxysporum* f.sp. *radicis-lycopersici* and *B. cinerea* (Table 2). Total growth inhibition of *O. novo-ulmi* was observed in the presence of compound **3**, which also proved to be the most effective derivative against *A. alternata* and *R. solani*. The two *Phytophthora* species were more sensitive to derivative **4** (Table 2). These results showed that the carboxyl group is a structural feature important for the antifungal activity of PCA.

It is well known that phytopathogenic fungi have developed several mechanisms that allow them to counteract the effects of antibiotic compounds produced by biocontrol agents. These responses include different defensive mechanisms (Duffy, Schouten, & Raaijmakers 2003). So far, the interaction between plant pathogenic fungi and phenazines has not been broadly investigated. This study gives the opportunity to formulate some hypotheses on the response of some pathogenic fungi to PCA. For instance, the data presented in this work lead us to hypothesise that *R. solani* has evolved a mechanism more specific for the detoxification of PCA than for the compounds **3** and **4**. Furthermore, the partial loss of activity against *F. oxysporum* f.sp. *radicis-lycopersici* could be associated with a particular function that PCA plays inside the fungal host cells.

## 3. Experimental

### 3.1. *General*

IR Spectra: Perkin-Elmer (Norwalk, CT) Spectrum One FT-IR spectrometer; neat, $\nu$ in $cm^{-1}$. UV Spectra: Perkin-Elmer Lambda 25 UV/Visible spectrophotometer in MeOH; $\lambda_{max}$ (log $\varepsilon$) in nm. $^1$H NMR Spectra: Bruker (Karlsruhe, Germany) spectrometer at 400 and 600 MHz; $\delta$ in ppm relative to each solvent used as internal standard, $J$ in Hz. ESI-MS: Agilent Quadrupole 6120 LC/MS (Waghaeusel-Wiesental, Germany) spectrometer; in $m/z$. Anal. and prep. TLC: silica gel ($SiO_2$; Kieselgel 60, $F_{254}$, 0.25 and 0.50 mm, respectively, Merck, Darmstadt, Germany) plates; the spots were visualised by exposure to UV radiation (254 nm) and/or by spraying first with 10% $H_2SO_4$ in methanol

and then with 5% phosphomolybdic acid in ethanol, followed by heating at 110°C for 10 min. Column chromatography (CC): $SiO_2$ column (Merck, Kieselgel 60, 0.063–0.200 mm).

### 3.2. *Bacterial strain and fungal isolates*

*Pseudomonas chlororaphis* subsp. a*ureofaciens* strain M71, a bacterium isolated from tomato rhizosphere (Puopolo, Raio, Pierson, & Zoina, 2011), was deposited in the herbarium of the Dipartimento di Arboricoltura, Botanica e Patologia Vegetale Universitá di Napoli Federico II, Portici, Italy. The bacterial strain was maintained in 40% glycerol at −20°C and was routinely grown on Nutrient Agar plates. Fungal isolates employed in this work belong to the collection of the Dipartimento di Arboricoltura, Botanica e Patologia Vegetale Universitá di Napoli Federico II, Portici, Italy, and of the Istituto per la Protezione delle Piante, CNR-Area di Ricerca di Firenze. Fungal isolates were stored in sterile tubes containing potato dextrose agar (PDA; Difco, Detroit, MI) and routinely grown on PDA plates.

### 3.3. *Production, extraction and purification of phenazines*

Strain M71 was grown for 1 week at 27°C in 4 L of PPMD, as previously reported (Wood, Gong, Daykin, Williams, & Pierson, 1997). The lyophilised material from the culture filtrates was dissolved in distilled water, acidified to pH 2 with concentrated HCl and extracted exhaustively with $CHCl_3$ ($3 \times 200$ mL). The organic extracts were combined, dried with $Na_2SO_4$, filtered and evaporated under reduced pressure. The brown oily residue (485.7 mg) was fractionated by CC on $SiO_2$ eluted with $CHCl_3$-*i*-PrOH 9:1 yielding four groups of homogeneous fractions. The residue of the first fraction yielded the main compound, identified as PCA (**1**), a homogeneous compound ($R_f = 0.69$, 106.5 mg). The residue of the second fraction (250 mg) was further purified by CC on $SiO_2$ eluted with $CHCl_3$-*i*-PrOH 95:5 yielding three groups of homogeneous fractions. The residue of the first fraction yielded a further amount (18.6 mg) of the main compound (**1**) as a homogeneous compound. The residue of the second and third fractions, which proved to be a mixture of two metabolites, was combined and further purified by prep. TLC ($SiO_2$; eluent $CHCl_3$-*i*-PrOH 95:5) to give 16 mg of **1** (total 141.1 mg, 35.2 mg $L^{-1}$) and another homogeneous compound (64 mg), identified as 2-OH P (**2**, $R_f = 0.35$, 16 mg $L^{-1}$).

#### 3.3.1. *Phenazine-1-carboxylic acid (**1**)*

IR: 2916, 1718, 1622, 1600, 1562, 1522 (Brisbane, Janik, Tate, & Warren, 1987): IR at 0.5% KCl: 3040, 3020, 1740, 1625, 1605, 1565, 1525); UV: 364 (3.81). $^1$H NMR (recorded in $CDCl_3$) and ESI-MS were previously reported (Raio et al., 2011).

#### 3.3.2. *2-Hydroxyphenazine (**2**)*

IR: 3328, 1647,1449,1411; UV: <220 (Pierson & Thomashow, 1992: UV/Visible, 30% $CH_3CN$: 372, 250). $^1$H NMR (recorded in $CD_3OD$) and ESI-MS were previously reported (Raio et al., 2011).

#### 3.3.3. *Phenazine-1-carboxymethyl (**3**)*

An ethereal solution of $CH_2N_2$ (300 µL) was added to PCA (**1**, 60 mg) dissolved in MeOH (2 mL). The reaction was carried out overnight at room temperature (r.t.) in the dark. The reaction was stopped by evaporation under a $N_2$ stream. The residue (63.2 mg) was

purified by prep. TLC ($SiO_2$; eluent $CHCl_3$-*i*-PrOH 95:5) to obtain 1-carboxymethylphenazine (**3**, 50 mg). IR: 1718, 1559, 1521, 1462; UV: 365 (4.12), 248 (4.82); $^1$H NMR, $CD_3OD$, $\delta$: 8.39 (d, $J = 8.7$, H-4), 8.34 (d, $J = 6$, H-2), 8.33 (d, $J = 8.7$, H-6), 8.23 (d, $J = 6.4$, H-9), 7.87 (m, H-3, H-7 and H-8), 4.11 (s, OMe); ESI-MS (+): 261 $[M + Na]^+$, 239 $[M + H]^+$.

### 3.3.4. *Phenazine-1-carboxamide (**4**)*

To phenazine-1-carboxymethyl (**3**, 15.2 mg) dissolved in MeOH (2 mL), 30% $NH_3$ (10 µL) was added. The reaction was carried out at r.t. overnight. After 24 h, the reaction was complete and the mixture was evaporated under reduced pressure. The residual crude oil (14.8 mg) was purified by prep. TLC ($SiO_2$, eluent $CHCl_3$-*i*-PrOH 95:5) to yield derivative **4** (11.4 mg) as a homogeneous compound. IR: 3327, 1694, 1524; UV: 366 (4.51), 248 (4.97); $^1$H NMR (recorded in $CDCl_3$) is very similar to that previously reported (Chin-A-Woeng et al., 1998); ESI-MS (+): 246 $[M + Na]^+$, 224 $[M + H]^+$.

### 3.3.5. *Phenazine-1-hydroxymethyl (**5**)*

$LiAlH_4$ (9 mg) was added to phenazine-1-carboxymethyl (**3**, 26 mg) dissolved in anhydrous ethyl ether (2 mL) by stirring with ice. After 30 min, 2 mL of EtOAc and 8 mL of distilled $H_2O$ were added. The solution was neutralised with 1 M HCl and then extracted with EtOAc (3 × 20 mL). The organic extracts were combined, dried ($Na_2SO_4$) and evaporated under reduced pressure. The oily residue (24.2 mg) was purified by prep. TLC ($SiO_2$, eluent $CHCl_3$-*i*-PrOH 9:1) to yield derivative **5** (17.2 mg). IR: 3280, 11524, 1425; UV: 362 (4.17), 250 (5.05). $^1$H NMR, $CDCl_3$, $\delta$: 8.32 (dd, $J = 7.4$, 2.2, H-4), 8.25 (dd, $J = 7.0$, 2.2, H-2), 8.15 (d, $J = 8.7$, H-6), 8.02 (d, $J = 6.8$, H-9), 7.96 (m, H-3, H-7 and H-8), 4.89 (s, $CH_2$-OH); ESI-MS (+): 233 $[M + Na]^+$, 211 $[M + H]^+$.

### 3.3.6. *Phenazine-1-acetoxymethyl (**6**)*

To phenazine-1-hydroxymethyl (8.2 mg) dissolved in pyridine (240 µL), $Ac_2O$ (240 µL) was added under stirring. The reaction was carried out at 80°C for 30 min. The reaction was stopped by the addition of MeOH and evaporation of the mixture by a $N_2$ stream. The residual crude oil (7.2 mg) was purified by prep. TLC ($SiO_2$, eluent $CHCl_3$-*i*-PrOH 9:1) to yield derivative **6** (6.9 mg) as a homogeneous compound. IR: 1744, 1526, 1463, 1228 ((Ichiro & Kan, 1964): $\nu_{max}^{nujol}$ ($cm^{-1}$): 1742, 1242); UV: 363 (4.06), 250 (4.76); $^1$H NMR, $CDCl_3$, $\delta$: 8.50 (br d, $J = 7.4$, H-4), 8.46 (br d, $J = 7$, H-6), 8.36 (d, $J = 8.4$, H-9), 7.95 (m, H-2, H-3, H-7, H-8), 6.00 (s, $CH_2O$), 2.21(s, MeCO); ESI-MS (+): 275 $[M + Na]^+$, 253 $[M + H]^+$.

## 3.4. In vitro *assays*

The sensitivity of 25 phytopathogenic fungal strains and oomycetes (listed in Table 1) to PCA and 2-OH P was assessed in an agar plate bioassay. A 5-mm-diameter plug was excised from the margin of a 7-day-old colony of each fungal strain and placed in the centre of a 60 mm-diameter petri plate containing PDA or PDA plates amended with three different concentrations (10, 25 and 50 µg $mL^{-1}$) of PCA and 2-OH P. The four derivatives of PCA (**3**, **4**, **5** and **6**) were evaluated against a set of 11 fungal strains at a concentration of 25 µg $mL^{-1}$ under the same experimental conditions described above. Each combination of fungal strain/amended PDA and fungal strain/non-amended PDA was duplicated in all the trials. Once inoculated, petri plates were incubated at 21°C for 6 days, and radial growth was measured at the end of the trial. Growth inhibition of fungal isolates by 2-OH P, PCA and its derivatives was quantified according to the following formula: $[(R_c - R_s)/$

$R_c$] %, where $R_c$ stands for the fungal colony radius on non-amended PDA plates while $R_s$ indicates fungal colony radius on amended PDA plates. The values 0% and 100% indicated no growth inhibition and complete growth inhibition, respectively. The experiment was replicated thrice. Results were analysed by ANOVA and the significance of differences was compared using Tukey's test.

### 3.5. *Assays in liquid culture*

This experiment was carried out in order to better determine the sensitivity to PCA of those fungi that were able to overgrow on PDA plates amended with 25 μg mL$^{-1}$ of this compound. *Phytophthora cinnamomi, P. ultimum* and *R. solani* were employed in this assay. Five tubes containing 40 mL of potato dextrose broth (PDB) only and five tubes containing PDB amended with 25 mg mL$^{-1}$ of PCA were inoculated with a 5-mm-diameter plug excised from the margin of 7-day-old colony of each fungal strain. Tubes were incubated on a rotary shaker (150 rpm) at 21°C for 6 days. At the end of the incubation period, mycelia were collected from each tube, dried under vacuum for 30 min and then weighed. The significance of differences between the average of mycelium weight from PDB and PCA-amended PDB was compared by Student's *t* test. At the same time, supernatants were collected in new tubes and extracted according to the procedure previously reported in order to survey if any PCA degradation had occurred in the presence of the growing fungi. Five uninoculated tubes containing PDB and PDB amended with PCA at 25 μg mL$^{-1}$, incubated at the same conditions reported above, were used as controls. The supernatants of strain M71 were extracted as previously reported, and the organic extracts were dried with $Na_2SO_4$, filtered and evaporated under reduced pressure. The extracts were resuspended in MeOH at a final concentration of 1 μg of extracts for 1 μL. A volume of 3 μL from each sample was further analysed by TLC ($SiO_2$, eluent $CHCl_3$-*i*-PrOH 95:5). The same volume of PCA solution (1 μg μL$^{-1}$) was loaded on TLC on silica gel to check for the presence of degradation products.

## 4. Conclusions

Data reported in this work show that the carboxyl group of PCA is a structural feature important for its antifungal activity, which remained substantially unaltered only in the derivatives **3** and **4**. Furthermore, this work provides useful information for the improvement of the applications of *P. chlororaphis* subsp. *aureofaciens* strain M71, describing the host range of plant pathogenic fungi sensitive to this compound.

## Supplementary material

$^1$H and $^{13}$C NMR and ESIMS spectra of compounds **1**–**6** are available online as Figures S1–S13.

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