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Synthesis, fungicidal activity and phloem mobility of phenazine-1-carboxylic acid-alanine conjugates

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ABSTRACT

Phenazine-1-carboxylic acid (PCA) is a natural product that has been proven effective against a number of soil-borne fungal phytopathogens and registered for biofungicide against rice sheath blight in China. In order to improve the phloem mobility of phenazine-1-carboxylic acid (PCA), four PCA derivatives were designed and synthesized by conjugating PCA with L-alanine methyl ester, D-alanine methyl ester, L-alanine and D-alanine respectively. *In vitro* and *planta* bioassays results showed that conjugates L-PAM and D-PAM exhibited higher fungicidal activities against *Rhizoctonia solani* Kuhn than PCA while L-PA and D-PA were less active than PCA. The concentration of conjugates in *Ricinus communis* phloem sap was determined by HPLC. The results showed that only L-PA exhibited phloem mobility among these conjugates, and its concentration in *Ricinus communis* phloem sap increased with the increase of time (the maximum concentration was 12.69 μM within 5 h). However, the results of pot experiments showed that L-PA and other conjugates didn't exhibited the inhibition for the growth of *Rhizoctonia solani* Kuhn in the lower leaves after treatment in the upper leaves of rice seedlings. This may be due to the poor plant absorbility for them or their too little amount of accumulation in the lower leaves.

1. Introduction

In recent years, phloem-mobile pesticides have attracted more and more attention because they are more economical and efficient than pesticides without phloem mobility [1–3]. Especially for fungicides, the development of phloem-mobile fungicides that can be applied to foliage to control root or vascular pathogens has long been highly desired [4]. Therefore, the development of pesticides with phloem mobility is of great significance to agricultural production.

The movement of xenobiotics within the phloem is associated with their physicochemical properties (octanol-water partition coefficients, Log K_{ow} and acid dissociation constant, p*K*a) and the plant parameters (plant size, pH of the phloem sap, and so on) [5–6]. There are also a number of xenobiotics whose transport in the phloem is carrier-mediated [7–12]. One efficient strategy for developing phloem-mobile pesticides is to conjugate an endogenous phloem mobile substrate, such as an amino acid or carbohydrate, with the active non-phloem-mobile pesticide molecules to make them have phloem mobility using the plant endogenous transporters [12]. For instance, ε-(2, 4-Dichlorophenoxyacetic acid)-L-lysine (2, 4-D-Lys), which is one of these derivatives, exhibits good phloem mobility, and its uptake is mediated by an active carrier system [7]. Another novel compound is fipronil-glucose conjugate (GTF) and the linking glucose can change fipronil into a phloem systemicity type, which is mediated by monosaccharide transporters [2,11].

Phenazine-1-carboxylic acid (PCA) is a natural product isolated from metabolites extracted from *phytopathogens* sp. M18 [13]. PCA has been proven effective against a number of soil-borne fungal phytopathogens and registered for biofungicide against rice sheath blight in China [14–16]. Phenazine-1-carboxamide, the derivative of PCA, is also a natural product isolated from metabolites of *Pseudomonas chlororaphis* PCL1391 [17]. Its fungicidal activity against *Rhizoctonia solani* Kuhn is five to ten times as much as that of PCA [17]. But neither of them has phloem mobility.

In order to improve the phloem mobility and bioactivities of PCA, PCA and Phenazine-1-carboxamide as the lead compounds, a series of PCA-amino acid ester conjugates were synthesized in our previous studies (including L-PAM and D-PAM) [18]. Unfortunately, although these conjugates exhibited excellent activities against *Rhizoctonia solani* Kuhn, all of them didn't have phloem mobility. It is probably because the transporters of amino acid can't recognize or transport these conjugates whose functional groups forming amino acid, α-amido and α-carboxy, have been binding. Consequently, the hydrolysis of these phenazine-1-carboxylic acid-amino acid ester conjugates and the

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evaluation of phloem mobility of phenazine-1-carboxylic acid-amino acid conjugates is our present work.

In this paper, we synthesized novel compounds L-PA and D-PA by the hydrolysis of L-PAM and D-PAM. Herein, we report their synthesis, fungicidal activities, phloem mobility in *Ricinus communis* L. seedlings and systemicity in rice seedlings.

2. Materials and methods

2.1. Chemicals

Reagents and anhydrous solvents were used as purchased without further purification. Melting points were determined on an XT4A digital micro melting point apparatus. TLC was taken on a silica gel plate (GF254, Qingdao, China). Silica gel (200–300 mesh) was used for flash column chromatography. NMR spectra were provided by a Bruker AVANCE III 600 (Brunker Corporation, Switzerland) spectrometer with tetramethylsilane (TMS) as the internal standard, and chemical shifts were recorded. The mass spectrographic analysis was recorded on a Waters ZQ4000 (Waters, MA, USA) with electron-spray ionization.

2.2. Plant materials

Castor bean seeds (*Ricinus communis* L.) were obtained from the Agricultural Science Academy of Zibo Shandong China and grown as previously described [4]. Then, six-day-old seedlings (the hypocotyl was about 20 mm length) were selected for further experiments. Rice seeds (Liangyou 287) were provided by Agricultural School, Yangtze University.

2.3. Test fungus

Rhizoctonia solani Kuhn, *Fusarium graminearum* Schw. and *Fusarium oxysporun* f. sp. *niveum* were provided by Department of Pathology, Agricultural School, Yangtze University.

2.4. General procedure for the preparation of L-PAM, D-PAM, L-PA and D-PA

Treatment of PCA (0.0025 mol) with oxalyl chloride (0.003 mol) and DMF as catalyst in CH_2Cl_2 solution at reflux temperature afforded intermediate **1** after evaporation of CH_2Cl_2 and excessive oxalyl chloride. L-PAM and D-PAM were then synthesized by adding corresponding alanine methyl ester (0.0025 mol) to intermediate **1** in CH_2Cl_2 solution, utilizing triethylamine (10 ml) as a base at 0 °C, and stirring overnight. Reacting corresponding Compound (L-PAM or D-PAM) with LiOH in methanol-water solution (LiOH 3.8 g, methanol 10 ml, water 40 ml) at room temperature for 10 h gave the compounds, L-PA and D-PA (Scheme 1).

2.4.1. L-2-[(phenazine-1-carbonyl) amino] propanoic acid (L-PA)

Yellow solid, yield 68%; mp 168–174 °C dec; ^{1}H NMR (600 MHz, CD_3OD) δ: 11.94 (s, 1H, N—H), 9.03–7.95 (m, 7H, Ar—H), 4.95 (m, 1H, C—H), 1.82 (d, J = 7.1 Hz, 3H, C-CH_3); ^{13}C NMR (600 MHz, DMSO-d_6) δ: 174.56 (COOH), 163.76 (C = O), 143.33, 143.06, 141.36, 140.49, 135.03, 133.90, 132.88, 134.31, 130.87, 129.74, 129.51 and 129.35 (Ar—C), 49.23 (CH), 18.60 (CH_3); HRMS (ESI) calcd for $C_{16}H_{13}N_3O_3$ $[M + H]^+$ 296.1036, found 296.1030.

2.4.2. D-2-[(phenazine-1-carbonyl) amino] propanoic acid (D-PA)

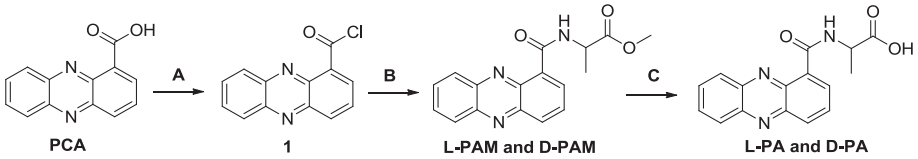
Yellow solid, yield 64%; mp 169–174 °C dec; ^{1}H NMR (600 MHz, CD_3OD) δ: 11.94 (s, 1H, N—H), 9.03–7.95 (m, 7H, Ar—H), 4.95 (m, 1H, C—H), 1.83 (d, J = 7.1 Hz, 3H, C-CH_3); ^{13}C NMR (600 MHz, DMSO-d_6) δ: 174.55 (COOH), 163.76 (C = O), 143.32, 143.05, 141.35, 140.48, 135.03, 133.89, 132.89, 132.31, 130.87, 129.73, 129.49 and 129.35 (Ar—C), 49.23 (CH), 18.60 (CH_3); HRMS (ESI) calcd for $C_{16}H_{13}N_3O_3$ $[M + H]^+$ 296.1034, found 296.1030.

2.5. In vitro assays of fungicidal activities

All the synthesized conjugates were evaluated for their fungicidal activities *in vitro* against *Rhizoctonia solani* Kuhn, *Fusarium graminearum* Schw. and *Fusarium oxysporun* f. sp. *niveum* by using a plate method (PDA medium). And PCA was used as a positive control. Fungicidal activities of conjugates were tested with the hyphae growth velocity assay [19]. The effective concentration of the sample causing a 50% inhibition of mycelial growth (EC_{50}) was determined. The commercial fungicidal agent phenazine-1-carboxylic acid, produced by Shanghai Nongle Biological Products Co., Ltd. China, was used as positive control. Mycelial discs (5 mm in diameter) of phytopathogenic fungi grown on PDA were cut from the margins of the colony and placed on the same medium containing different concentrations of the sample. A negative control was maintained with sterile water mixed with PDA medium. Each treatment had three replicates. The diameter of colony growth was measured when the fungal growth in the control had completely covered the Petri dishes. The inhibition percentage of mycelial growth was calculated as follows: Mycelial growth inhibition (%) = $(D_a - D_b)/D_a \times 100$ where D_a represents control colony diameter and D_b represents treated colony diameter. The colony diameter is in millimeters. All statistical analysis was performed using EXCEL 2010 software. The log dose-response curves allowed determination of the EC_{50} for the fungi bioassay according to probit analysis. The 95% confidence limits for the range of EC_{50} values were determined by the least-square regression analysis of the relative growth rate (% control) against the logarithm of the compound concentration.

2.6. Phloem sap collection and analysis

The phloem sap collection method was similar to these papers described [4,7,20]. The cotyledons were incubated in buffered solution containing 20 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), 0.25 mM $MgCl_2$, and 0.5 mM $CaCl_2$ at pH 5.5, and every target compound was used at 200 μM in these experiments. The phloem sap was analyzed by high-performance liquid chromatography (HPLC) after dilution with water (phloem sap/water, 1:1, v/v). SHIMADZU LC-10Avp HPLC system with a vacuum degasser and an ultraviolet – visible (UV – vis) detector were employed for analysis. A C18 reversed-phase column (5 μm, 250 × 4.6 mm inner diameter) was used and maintained at 45 °C. The mobile phase consisted of acetonitrile and water (70:30, v/v), at a flow rate of 0.8 ml min^{-1}, and the injection volume was 20 μL. The absorbance wavelength was 248 nm. A series of standard solutions of title compounds (0.78, 1.56, 3.13, 6.25, 12.5, 25, and 50 μM) for linearities were prepared in acetonitrile. The linear equation of L-PA was y = 43,869 × + 823.71 (r = 0.9942).



Scheme 1. Preparation of target compounds (L-PAM, D-PAM, L-PA and D-PA). Reagents and conditions: (A) oxalyl chloride, DMF, CH_2Cl_2, reflux, 4 h; (B) alanine methyl ester, triethylamine, CH_2Cl_2, 0 °C to room temperature, overnight; (C) LiOH 3.8 g, methanol 10 ml, water 40 ml, room temperature, 10 h.

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