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Biocontrol of *Lysobacter enzymogenes* CQ18 against the tobacco powdery mildew fugus, *Erysiphe cichoracearum*

Hongjun Yang[1†], Liyuan Peng[1,2†], Zhimo Li[3], Chunyang Huang[3*] and Jianguo Huang[1*]

Abstract

Background Powdery mildew is a common leaf disease of crops worldwide. A large quantity of chemical fungicides is used to control this disease in horticulture and agriculture, producing serious safety and environmental problems. To suppress this disease in safe and environment-friendly ways, the biocontrol of a self-isolated new strain of *Lysobacter enzymogenes* (CQ18) was studied against flue-cured tobacco powdery mildews.

Results *L. enzymogenes* CQ18 produced chitinase, protease, β-1,3-glucanase, phosphatase, and siderophore, which may enable this biocontrol bacterium to degrade pathogen cell membranes and walls and deprive pathogens of iron. HPLC/MS analysis identified 14 antifungal metabolites present in *L. enzymogenes* CQ18 fermentation liquid (LEFL), which were grouped into organic acids, azoles, and pyrimidines. The variable targets in or on pathogen cells and combinative effects of these multiple metabolites may potently suppress the powdery mildew and be less likely to make *Erysiphe cichoracearum* develop resistance. LEFL was rich in L-pyroglutamate. Both LEFL and L-pyroglutamate inhibited the germination of *E. cichoracearum* conidia in vitro and reduced the powdery mildew index in the greenhouse and field. L-Pyroglutamate at a concentration of 0.50% achieved the same control efficacy as the chemical fungicide triadimefon (91–94%).

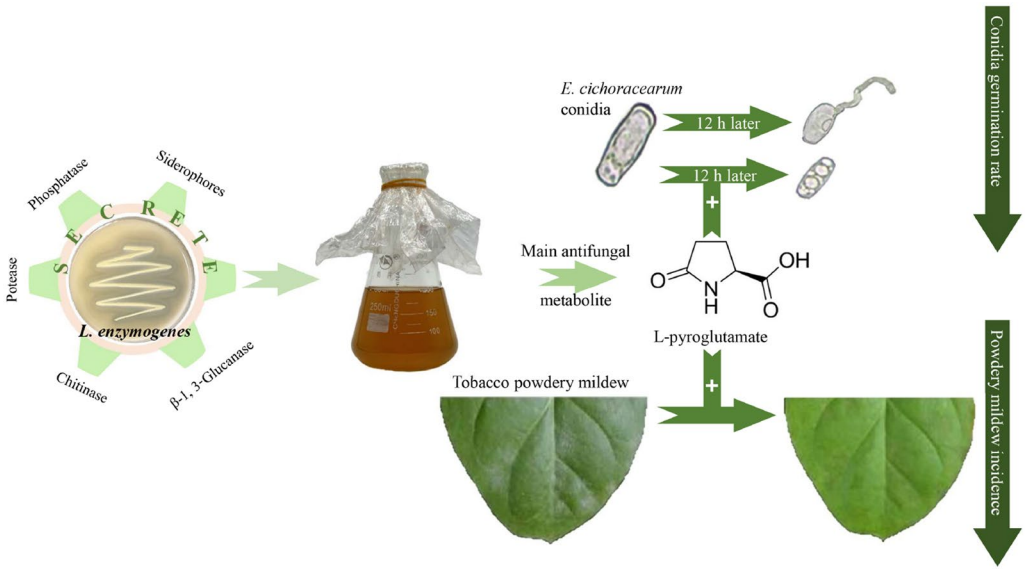
Conclusions *L. enzymogenes* CQ18 and the metabolite L-pyroglutamate effectively controlled flue-cured tobacco powdery mildew. *L. enzymogenes* CQ18 grows rapidly and is resilient to adversity. L-Pyroglutamate has no toxicity to humans and is easy to synthesize at a low cost. Both show potential use in controlling plant powdery mildews.

Keywords Powdery mildew, *Lysobacter enzymogenes*, Biological control, L-Pyroglutamate

†Hongjun Yang and Liyuan Peng contributed equally to this work.

*Correspondence:
Chunyang Huang
hcy_0701@126.com
Jianguo Huang
huang99@swu.edu.cn
Full list of author information is available at the end of the article

Graphical Abstract



Introduction

Powdery mildew is a common and visible leaf disease worldwide, which causes great yield and economic losses every year in agriculture and horticulture [1, 2]. Due to repeated incidences within a crop growing season, a large quantity of chemical fungicides has to be frequently used to control this disease [3, 4]. As a result, the pathogens easily develop resistance to chemical fungicides, leading to low control efficacy or failure of the fungicides [5, 6]. The continuous development of new fungicides is necessary to effectively control powdery mildews. However, it often takes a long time for scientists to develop new chemical fungicides. The frequent use of chemical fungicides in large quantities also produces serious environmental and food safety problems.

Biological control is safe for humans and friendly to environments, which provides a new alternative to control crop powdery mildew. For example, *Bacillus subtilis* suppressed lettuce powdery mildew incited by *Podosphaera fusca* [7]. *Bacillus amyloliquefaciens*, an endophyte bacterium, had a broad antimicrobial spectrum with high biocontrol efficacy, enhanced the ability of tobacco plants to resist powdery mildews, and thus greatly reduced the incidence of tobacco powdery mildews caused by *Erysiphe cichoracearum* [8–11]. *Pseudomamma aphid is* significantly inhibited the conidial germination of *Podosphaera xanthii*, a pathogen fungus that incites the powdery mildew of many plants by parasitism [12]. Similarly, *Tilletiopsis pallescens*, *Simplicillium aogashimaense*, and *Trichothecium roseum* significantly inhibited the growth and propagation of *Blumeria graminis f.* sp. *tritici*. As a result, they impaired colony formation on wheat leaves and effectively suppressed wheat powdery mildews [13–16]. However, limited biocontrol microbes are used in powdery mildew management. It is necessary to discover more microbes effective in controlling this foliar disease.

Lysobacter spp., a Gram-negative bacterium with gliding motility, is ubiquitous in soil, sludge, and freshwater. Some members in this genus have long been studied for their ability to inhibit plant pathogens [17]. For instance, *L. enzymogenes* C3 can suppress many plant fungal diseases in both greenhouse and field experiments [18, 19]. Upon exposure to *L. enzymogenes* C3, nematode juveniles were quickly immobilized and then lysed within several days, indicating the production of toxins and extracellular lytic enzymes by this biocontrol bacterium [20]. *L. enzymogenes* 3.1T8 antagonized *Pythium aphanidermatum* due to the synthesis of polyketide synthase [21]. Chitinase, lipases, and protease produced by *L. capsici* PG4 are involved in the degradation of fungal cell membranes and walls, inhibiting the growth of many phytopathogen fungi [22]. As a result, *L. capsici* PG4 significantly decreased the incidence of tomato phytophthora rot in greenhouses. Taken together, some members of *Lysobacter* antagonize plant pathogens in many

different ways, including the production of hydrolases, antimicrobial substances, and or the induction of plant defense response [20, 23, 24]. These studies rarely focus on crop powdery mildew. Evaluating the control efficacy of *Lysobacter* spp. against powdery mildew can provide farmers with safe and eco-friendly options in this disease management.

A self-isolated new strain of *L. enzymogenes* CQ18 showed potent inhibitory effects on a variety of culturable phytopathogen fungi in pure culture. However, whether this biocontrol bacterium can control powdery mildew is unknown. The objectives of this study were to (i) detect the fungal cell-wall-degrading hydrolases produced by this bacterium; (ii) identify the antifungal metabolites present in LEFL by high-performance liquid chromatography/mass spectrometry (HPLC/MS), and (iii) evaluate the biocontrol efficacies of this bacterium and L-pyroglutamate (an antifungal metabolite present in LEFL) against flue-cured tobacco powdery mildew in both greenhouse and field.

Materials and methods

Bacterial strain

L. enzymogenes CQ18 (previously named the strain of HYP18, accession number in the China General Microbiological Culture Collection Center was MT377819) used in the current experiments was originally isolated from a purplish soil in 2018. After growing the bacteria on Luria–Bertani agar (LB) at 25 ℃ in the dark for 5 days, the bacteria were transferred to sterile deionized water and diluted to approximately 1×10^3 cells mL^{-1} as a liquid inoculant in the follow-up experiments.

Determination of extracellular lytic enzymes and siderophores

L. enzymogenes CQ18 was individually grown on the agar mediums containing colloidal chitin, skimmed milk powder, β-1,3-glucan, lecithin, and chrome azurol S at 25 °C for 3–5 days in the dark to determine the production of chitinase, protease, β-1,3-glucanase, phosphatase, and siderophores [25].

Detection of antimicrobial metabolites in LEFL

One mL of liquid inoculant (1×10^3 cells mL^{-1}) was inoculated in a 1000-ml flask containing 500 mL of sterilized nutrient solution (NS; 5.0 g beef extract, 10.0 g peptone, 5.0 g NaCl, 1000 mL deionized water, pH 7.0) and then incubated at 25 °C with constant agitation at 150 rpm in the dark for 5 days in the dark. Twenty-five mL of LEFL was passed through a 0.22-μm film to remove bacterial cells and other solids. 25 mL cell-free LEFL was dried at − 18 ± 2 °C using a freezer (Haier, Qingdao, China). The residues were dissolved in 5.0 mL of 5% methanol for the detection of metabolites by HPLC/MS (Thermo Electron, San Jose, CA, USA) at Morjorbio Company, Shanghai, China.

The liquid chromatography was performed using a C^{18} analytical column ACQUITY UPLC HSS T3 (100 mm × 2.1 mm; Waters, Milford, USA) with 95% water + 5% acetonitrile (containing 0.1% formic acid) and 47.5% acetonitrile + 47.5% isopropanol + 5% water (containing 0.1% formic acid) as mobile phase, respectively, at 0.4 mL min^{-1} of speed. The column temperature was 40 ℃ and 20 μL of samples were injected.

For MS analysis, an electron impact (EI) ionization source was used with an ion spray voltage 5500 V, capillary temperature 550 ℃, and a scan range of 35–500 m/*z* at a scanning interval of 0.30 s in the scan acquisition mode. N_2 was used as the nebulizer, heater, curtain, and collision gas. An Abf Converter, MS-DIAL [26], Shimadzu offline software, and the Fiehn library were used for peak data processing (including raw extraction, identification, and area integration to remove metabolites less than threefold blank), baseline filtering and calibration, and deconvolution analysis [27]. Following are the setting parameters: 0.5 sigma window value, 5000 EI spectra cut-off, 0.5 min retention time tolerance, 0.5 Da m/*z* tolerance, 70% EI similarity cut-off, and 70% identification score cut-off. The alignment settings were: 0.075 min for retention time tolerance and 0.5 for the retention time factor [28]. The matched substances were retained based on a total similarity filter ≥ 800, fill ≥ 0.5%, fragment presence ≥ 800, and unmatched metabolites were removed. The metabolome analysis was performed using three replicate fermentation broths with the same conditions.

Conidial germination test

Conidia of *E. cichoracearum* were collected from naturally infected leaves of tobacco plants in a field and identified using conidial characteristics [29]. Source leaves were shaken 24 h before harvesting the conidia to dislodge old ones and ensure the inoculant with high viability. The conidia were collected from mildewed leaves in a sterile beaker by violently shaking. They were then immediately spread on the sterile agars containing the cell-free LEFL at the concentrations of 0, 2, 4, 8, 16, and 32 mL L^{-1}, respectively, and incubated at 25 °C for 24 h [10]. The purpose to use the cell-free LEFL in this investigation was to understand the inhibitory effect of hydrolases and antifungal metabolites against the germination of *E. cichoracearum* conidia and at the same time to avoid the interference during microscopic observation. There were triplicate plates for each LEFL concentration and the experiment was performed twice. After incubating the plates at 25 ℃ for 12 h in darkness, three 1 × 1 cm agar pieces (the number of conidia on each piece ≥ 50) were

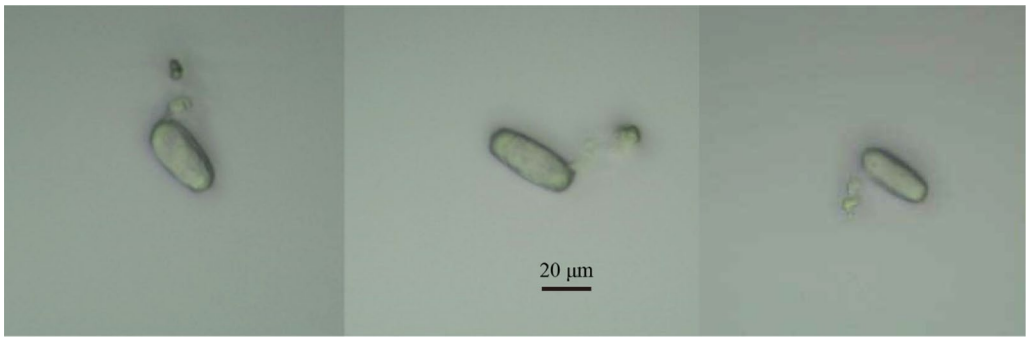


Fig. 1 Microscope images of germinated *Erysiphe cichoracearum* conidia after 12 h of dark incubation on water agar plate

randomly sampled from each plate for the observation of conidial germination using a light microscope (Olympus, Hamburg, Germany) (Fig. 1). The length of the germ tube exceeding half of the short radius of the conidia was regarded as germinated. Germination rate (%) = (number of germinated conidia / total number of observed conidia) × 100%.

HPLC/MS analysis found that the LEFL was rich in L-pyroglutamate. Some other *L. enzymogenes* stains can also produce this metabolite, which has been reported to have antifungal activity [30, 31]. Taking into account easy synthesis at low cost and non-toxicity to humans and animals [32], we further evaluated the influence of this metabolite on the conidial germination in the same way but using L-pyroglutamate (0, 1, 2, 4, 8, and 16 mg mL^{-1}) instead of the LEFL.

Efficacy of LEFL and L-pyroglutamate against powdery mildew

Greenhouse test

During the growing season of flue-cured tobacco (var. K326), 45-day-old plant seedlings were grown from seeds in plastic pots (one seedling per pot) and arranged at random on the benches in a greenhouse with natural temperature and light at Zunyi Tobacco Company, China (27.79°N, 107.68°E). The autoclaved solution containing 0.1% agar and 0.0025% Tween-20 (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was added to the beaker with conidia collected from tobacco leaves (see "Conidial germination test" section). This solution can well disperse the conidia and keep their viability for 4 h without an apparent decrease [33, 34]. The conidial inoculant (3×10^7 conidia mL^{-1}) was sprayed onto the whole plant until runoff on each leaf. The inoculated plants were grown in the greenhouse until powdery mildew colonies became visible on the leaves (about a week after inoculation). Then, spray treatments, including deionized water, 1000-time-diluted triadimefon solution, LEFL (1×10^{10} cells mL^{-1}), and aqueous solutions containing L-pyroglutamate at the concentrations of 0.125, 0.25, and 0.50% (all of them contained 0.01% of Tween 20), were applied on whole plants like the conidial inoculation [8, 35]. The experiment was carried out twice using a complete random design with four replicates for each treatment (10 pots per replicate). The colonies on each plant were counted before and after the 1st, 3rd, 5th, and 7th days of spray treatments. The colony counts of each plant were summed for data analysis.

Field experiment

Field experiments were carried out at Zunyi on June 5–17, 2021, and June 15–27, 2022 in tobacco (var. K326) fields (27.78°N, 107.66°E). The local climate is dominated by the Pacific monsoon with a mean annual temperature of 18 °C (6 °C in January–34 °C in August), a mean annual rainfall of 1400 mm (approximately 80% of the rainfall is received in April–August), and a mean annual sunshine of 1147 h. When powdery mildew colonies were visible on the leaves, spray treatments (i.e., CK, triadimefon, LEFL, and L-pyroglutamate at a concentration of 0.50%) were applied as described in the greenhouse test. The experimental plots (7×7 m) were randomly arranged with four replicates for each treatment. There were 73 tobacco plants in each plot.

Disease severities were daily rated for 10 consecutive days on a 0 to 9 scale, where 0 = no infection, 1 = 1–5%, 3 = 6–10%, 5 = 11–20%, 7 = 21–40%, 9 > 40% leaf area infected by powdery mildew [36]. Disease index $= \frac{\Sigma\ disease\ scale \times number\ of\ corresponding\ leaves}{maximum\ disease\ scale \times total\ number\ of\ leaves} \times 100$ [37].

Disease control efficacy (%) $= \frac{disease\ index\ of\ control - disease\ index\ of\ treatment}{disease\ index\ of\ control} \times 100$ [38].

Data treatment

The data shown in this paper were the averages of two experiments, including in vitro conidial germination and disease control experiments in the greenhouse and field. SPSS 23.0 software (SPSS Inc., Chicago, IL, USA) was

used to analyze one-way variance (ANOVA) of treatment effects on conidial germination rate in vitro, and two-way variance on the colony counts in the greenhouse and disease index and control efficacy in the field. Differences at the level of $P \leq 0.05$ in the least significant difference (LSD) test were considered significant.

Results

Lytic enzyme and siderophore productions

When *L. enzymogenes* CQ18 was grown on the agar plates individually containing colloidal chitin, skimmed milk powder, β-1,3-glucan, lecithin, and chrome azurol S, clear or colorful halos were observed surrounding the bacterial colonies (Fig. 2). So, the bacteria produced chitinase, protease, glucanase, phosphatase, and siderophores.

Antifungal metabolites present in LEFL

HPLC/MS analysis identified 14 antifungal metabolites present in LEFL, which were attributed to organic acids, azoles, and pyrimidines (Table 1). Organic acids included L-pyroglutamate, N-ribosylhistidine, N-undecylbenzenesulfonic acid, gentisic acid, 4-formylsalicylic acid, P-salicylic acid, hexyl salicylic acid, 13-oxo-9,11-tridecadienoic acid, 12-oxo-2,3-dinor-10,15-phytodienoic acid, 11-eicosenoic acid, and 4-(3-pyridyl)-3-butenoic acid. Nanaomycin belonged to azoles. Thymine, 3'-amino-3'-deoxythymidine, cytosine, and N-formyl-4-amino-5-aminomethyl-2-methylpyrimidine were attributed to pyrimidines. Among these soluble metabolites, L-pyroglutamate is noteworthy due to its high concentration in the LEFL, easy synthesis at a low cost, and non-toxic to humans and animals.

Antagonisms of LEFL and L-pyroglutamate against conidial germination

The rate of conidial germination on the agar plates logarithmically decreased with the concentrations of LEFL ($y = -44.7\ln(x) + 18.78$, $n = 6$, $r^2 = 0.953^{**}$) and L-pyroglutamate ($y = -9.371\ln(x) + 5.655$, $n = 6$, $r^2 = 0.921^{**}$; Table 2). The germination rate of *E. cichoracearum* conidia was 69.53% on the control agar and decreased to 2.10% with LEFL at a concentration of 32 mL L^{-1} and 0.35% with L-pyroglutamate at 16 mg L^{-1}. Based on the virulent equations established, the half inhibitory concentration against conidial germination (IC_{50}) was 5.13 mL L^{-1} for LEFL and 1.07 mg L^{-1} for L-pyroglutamate.

Efficacy of LEFL and L-pyroglutamate against tobacco powdery mildew in the greenhouse

The number of mildew colonies on the tobacco leaves varied in the sequence: CK > 0.125% L-pyroglutamate > LEFL > triadimefon and 0.25% L-pyroglutamate (no significant difference between triadimefon and 0.25% L-pyroglutamate) > 0.50% L-pyroglutamate (Table 3).

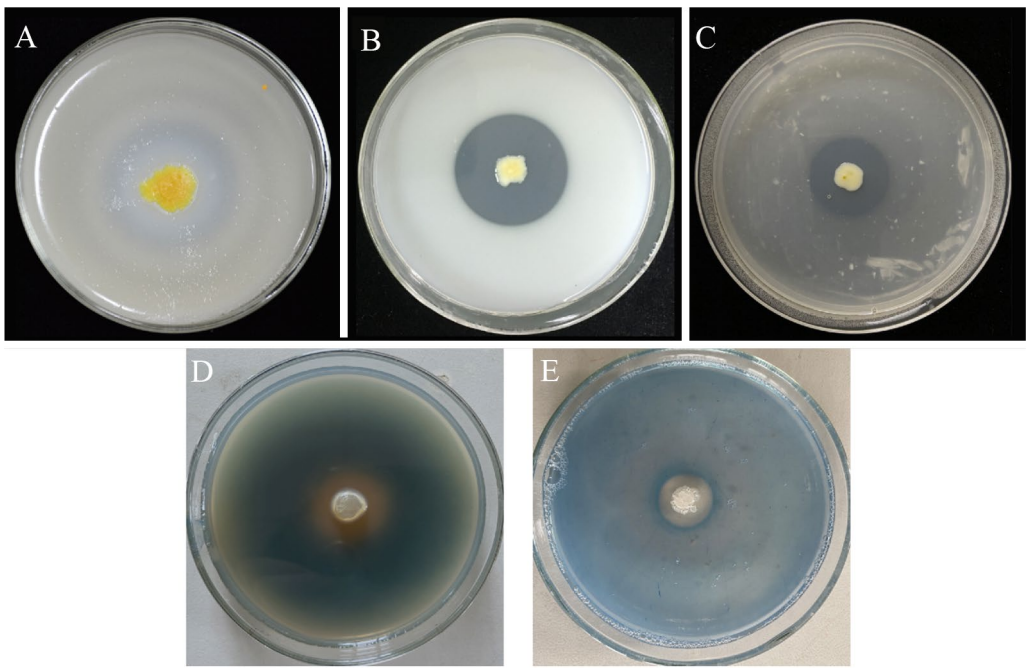


Fig. 2 *L. enzymogenes* CQ18 was individually grown on the agar mediums containing lecithin, skimmed milk powder, colloidal chitin, β-1,3-glucan, and chrome azurol S at 25 °C (**A** lecithin; **B** skimmed milk powder; **C** colloidal chitin; **D** β-1,3-glucan; **E** chrome azurol S)

Table 1 Antifungal metabolites detected in the *L. enzymogenes* CQ18 fermentation liquid

Groups	Metabolites	Formula	Functions
Organic acids			
	L-Pyroglutamic acid	$C_5H_7NO_3$	Antifungal activity [53, 54]
	Gentisic acid	$C_7H_6O_4$	Activation of plant defenses [56, 57]
	N-Undecylbenzenesulfonic acid	$C_{17}H_{28}O_3S$	Inhibition of fungal mycelial growth [48]
	N-Ribosylhistidine	$C_{11}H_{17}N_3O_6$	Inhibition of fungal mycelial growth [48]
	Hexyl salicylic acid	$C_{13}H_{18}O_3$	Antimicrobial activity [49–51]
	4-Formylsalicylic acid	$C_8H_6O_4$	Antimicrobial activity [52]
	P-Salicylic acid	$C_7H_6O_3$	As a module in activating disease resistance [58]
	13-Oxo-9,11-tridecadienoic acid	$C_{13}H_{20}O_3$	Antifungal activity [59]
	12-Oxo-2,3-dinor-10,15-phytodienoic acid,	$C_{16}H_{24}O_3$	Antifungal activity [60]
	11(Z)-Eicosenoic acid,	$C_{20}H_{38}O_2$	Damage to fungal cell membranes [45]
	4-(3-Pyridyl)-3-butenoic acid	$C_9H_9NO_2$	antifungal activity [61]
Quinone			
	Nanaomycin	$C_{16}H_{14}O_6$	Broad-spectrum antibiotic (bacteria and fungi)[62]
Pyrimidines			
	N-Formyl-4-amino-5-aminomethyl-2-methylpyrimidine	$C_7H_{10}N_4O$	Fungicidal activity [63]
	3′-Amino-3′-deoxythymidine	$C_{10}H_{15}N_3O_4$	Broad-spectrum antibiotic (bacteria and fungi) [64]
Σ	14		

Table 2 Influence of *L. enzymogenes* CQ18 fermentation liquid and L-pyroglutamate on the germination of *E. cichoracearum* conidia

LEFL			L-Pyroglutamate		
Concentrations (mL L^{-1})	Germination rate (%)	Virulent equation	Concentrations (mg L^{-1})	Germination rate (%)	Virulent equation
0	69.53 ± 10.87	$y = -44.7\ln(x) + 18.78$ $R^2 = 0.953^{**}$ $IC_{50} = 5.13$	0	69.53 ± 10.87	$y = -9.371\ln(x) + 5.655$ $R^2 = 0.921^{**}$ $IC_{50} = 1.07$
2	68.17 ± 10.31		1	68.17 ± 10.31	
4	51.88 ± 13.33		2	31.22 ± 11.15	
8	20.04 ± 10.13		4	6.34 ± 8.63	
16	6.15 ± 8.09		8	2.15 ± 3.05	
32	2.01 ± 3.58		16	0.35 ± 0.14	

IC_{50} half inhibitory concentration. *LEFL L. enzymogenes* CQ18 fermentation liquid

Table 3 Effects of foliar spray treatments on the number of colonies of powdery mildew on flue-cured tobacco leaves (no. per plant) in the greenhouse

Days after spays	CK	L-Pyroglutamate (%)			LEFL	Triadimefon
		0.125	0.25	0.50		
0	125 ± 5.91dA	123 ± 8.70aA	130 ± 10.01aA	124 ± 4.83aA	127 ± 6.90aA	122 ± 12.96aA
1	147 ± 9.00cA	84 ± 7.39cB	22 ± 7.48bD	5 ± 1.83bE	39 ± 6.58bC	24 ± 4.86bD
3	152 ± 10.15cA	87 ± 6.98cB	20 ± 3.92bD	9 ± 4.40bE	43 ± 7.39bC	27 ± 5.80bD
5	165 ± 5.35bA	90 ± 8.60cB	26 ± 7.09bD	8 ± 1.41bE	40 ± 6.32bC	25 ± 6.08bD
7	177 ± 3.77aA	102 ± 3.40bB	23 ± 5.72bD	7 ± 1.71bE	39 ± 6.50bC	25 ± 9.63bD

CK control, LEFL *L. enzymogenes* CQ18 fermentation liquid. The colonies were counted on whole plant leaves. In each row means followed by different capital letters are significantly different at $P \leq 0.05$ among the treatments. In each column, means followed by different lowercase letters are significantly different at $P \leq 0.05$ during observation days (least significant difference (LSD) multiple range test)

Moreover, the number of mildew colonies in the water and 0.125% L-pyroglutamate spray treatments increased with time but changed little, if any at all, in the other spray treatments (including L-pyroglutamate at concentrations of 0.25% and 0.5%, LEFL, and triadimefon) within the 7-day observation period.

Efficacy of LEFL and L-pyroglutamate against plant powdery mildews in the field

The mean disease index of powdery mildew was highest in the water spray control (24.30 ± 11.47), followed by LEFL (6.58 ± 3.37), and lowest with triadimefon and L-pyroglutamate (3.69 ± 3.35 and 3.45 ± 3.41, respectively; Fig. 3A). The disease indexes increased with time in the water spray control but changed little in the spray treatments of triadimefon, LEFL, and L-pyroglutamate during the 10-day observation period. As a result, the means control efficacy was 91.04 ± 2.92% for triadimefon and 92.12 ± 2.93% for L-pyroglutamate achieved (no significant difference between these two treatments), which were significantly higher than LEFL (78.66 ± 4.79%; Fig. 3B).

Discussion

Inhibitory effect of L. enzymogenes CQ18 against tobacco powdery mildew

Lysobacter species suppressed many plant diseases incited by bacteria, fungi, and nematodes [39–41]. Herein, LEFL (*L. enzymogenes* CQ18 fermentation liquid) greatly inhibited conidial germination and controlled powdery mildews of flue-cured tobacco in both greenhouse and fields although it was not as efficient as triadimefon, the chemical fungicide widely used to treat powdery mildews in the local area. It was worthwhile to further understand the mechanisms of this biocontrol bacterium employed in suppressing this foliar disease.

Production of extracellular enzymes and siderophore by L. enzymogenes CQ18

L. enzymogenes CQ18, like other *Lysobacter* species, produced extracellular chitinase, β-1,3 glucanase, phosphatase, and protease on agar plates. If the phenomena occur when contacting with powdery mildew colonies, the bacteria may degrade the pathogen cell membranes and walls. A similar antifungal mechanism was also employed by *L. enzymogenes* C3 and 3.1T8 against *Fusarium graminearum*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Didymella lycopersici*, *Cladosporium fulvum*, *Corynespora cassiicola*, *Verticillium albo-atrum*, and *Phytophthora capsici* [18, 21]. In addition, *L. enzymogenes* CQ18 produced siderophores, which may deprive the pathogen of iron inhibiting mycelial growth [42]. The presence of iron in cells may also induce *L. enzymogenes* CQ18 to synthesize antibiotic compounds because Fe^{3+} is a cofactor of some antimicrobial proteins involved in the inhibition of pathogens [43].

Antifungal metabolites present in LEFL (*L. enzymogenes* CQ18)

LEFL was rich in antifungal metabolites, which was consistent with other *L. enzymogenes* strains [44]. The antifungal metabolites produced by this bacterium were attributed to organic acids, quinone, and pyrimidines. Although these metabolites identified by HPLC/MS are not novel, they greatly varied in antifungal activities

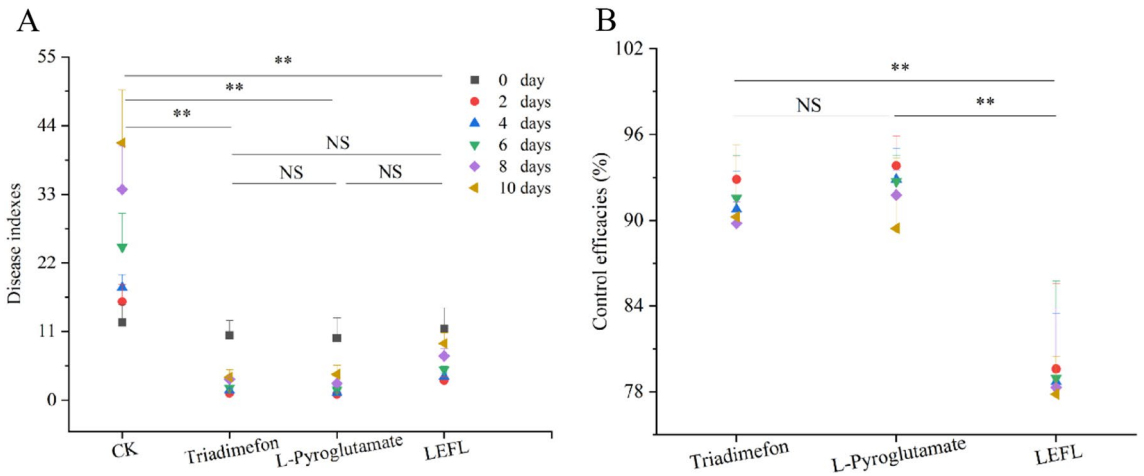


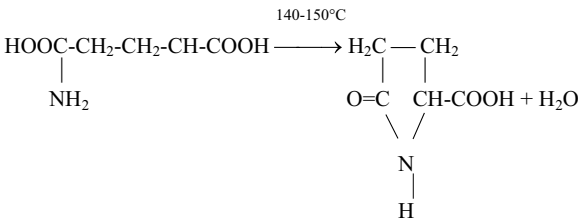
Fig. 3 The control efficacies of triadimefon, *L. enzymogenes* CQ18 fermentation liquid (LEFL), and L-pyroglutamate against tobacco powdery mildew in the field. ** above the lines represent significant differences ($P < 0.01$). NS represent no significant differences ($P > 0.05$)

(including interference with cell membrane synthesis, protein denature, mycelial growth inhibition, plant defense induction, etc.) and the targets on or in pathogen cells. For example, eicosanoids in the organic acid group can damage fungal cell membranes, leading to the leakage of cell inclusions [45]. Quinone blocks the biosynthesis of ergosterols, an important component of fungal cell membranes [46]. Upon exposure to pyrimidines, protein biosynthesis in fungal pathogen cells is inhibited [47]. The organic acids, such as gentisic acid, N-ribosylhistidine, and N-undecylbenzenesulfonic acid detected in LEFL slowed hyphal growth by multiple mechanisms [48]. Salicylic acids can effectively inhibit fungal growth at low concentrations and kill the pathogen fungi at higher concentrations [49]. Da Rocha Neto et al. (2015) reported that the solution containing salicylic acid in vitro inhibited the germination of *P. expansum* spores and reduced the incidence of blue mold on apple leaves even at low concentrations [50]. Apart from an effective inhibitory effect on fungal growth, salicylic acid is also an important hormone in flowering plants, which is synthesized in either the cinnamic acid or the chorismate pathways [51]. The most well-known role of this plant hormone is to induce plant defense responses [52]. The underlying mechanisms were owing to the deposition of callose plugs (reinforce plant cell walls), the accumulation of pathogenesis-related proteins (induce toxicity in microbes upon contact), and the synthesis of hydrogen peroxide (initiate hypersensitive responses). Thus, the variable targets and combinative effects of multiple metabolites produced by *L. enzymogenes* CQ18 may be beneficial to effectively suppressing tobacco powdery mildew and decreasing the probability of pathogens developing resistance.

Inhibitory effect of L-pyroglutamate against tobacco powdery mildew

Like some other *L. enzymogenes* strains and biocontrol bacteria [31], *L. enzymogenes* CQ18 produced L-pyroglutamate (also named 5-oxoproline, 5-oxopyrrolidine-2-carboxylic acid, L-5-pyrrolidone-2-carboxylic acid or (S)-(-)-2-pyrrolidone-5-carboxylic acid). This metabolite showed antagonistic activity against many phytopathogen fungi such *Pseudoperonospora cubensis*, *Plasmopara viticola*, *Phytophthora infestans*, *Phytophthora capsici Leonian*, *Phytophthora infestans*, and *Candida* spp. [53, 54]. Taking into account the addition to cosmetics in a large quantity for moisturizing and whitening the skin, L-pyroglutamate would thus be safe for humans and animals. Therefore, we further investigated the antagonism of this metabolite against the germination of *E. cichoracearum* conidia in vitro and the suppression against flue-cured tobacco powdery mildew in both the greenhouse and the field. The IC_{50} (half inhibitory concentration) of L-pyroglutamate against the conidial germination was 1.07 mg L^{-1}, which should be much higher than LEFL (IC_{50} = 5.13 mL L^{-1}) if calculated as the dry weight of active ingredients. The coexistence of multiple antifungal ingredients could make the LEFL effectively inhibit the germination of the pathogen conidia. However, tobacco powdery mildew was also effectively suppressed by increasing the concentration of L-pyroglutamate. Subsequently, L-pyroglutamate at a concentration of 0.5% achieved a control efficacy similar to 1000-time-diluted triadimefon.

L-Pyroglutamate is often synthesized from 2 L-glutamate molecules by removing a water molecule at 140–150 °C:



Therefore, L-pyroglutamate is easy to synthesize at a low cost. The complex of L-pyroglutamate with copper or zinc is more toxic to phytopathogen cells [53]. Microbial secondary metabolites are important sources of antibiotics, pharmaceuticals, and insecticides. According to statistics, at least 70% of current medical drugs come from secondary metabolites and their derivatives of microorganisms [55]. L-Pyroglutamate and its derivatives may thus be worthwhile to develop as new fungicides.

Conclusion

L. enzymogenes CQ18 and its metabolite L-pyroglutamate inhibited the germination of *E. cichoracearum* conidia and suppressed the powdery mildew of flue-cured tobacco in both the greenhouse and the field. L-pyroglutamate at a proper concentration achieved the same control efficacy as the chemical fungicide triadimefon. The underlying mechanisms employed by *L. enzymogenes* CQ18 in suppressing the powdery mildews could be the production of hydrolases, siderophores, and antifungal metabolites. Our study provided promising use of

L. enzymogenes CQ18 and L-pyroglutamate in controlling powdery mildews.

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Author contributions
HY: investigation, data curation, carried out the experiments, writing—original draft, validation. LP: greenhouse experiments, data collection and statistical analysis, writing—original draft. ZL: field investigation and experiments. CH: conceptualization, writing—review and editing, validation. JH: conceptualization, writing—review and editing, validation.

Declarations

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author details
[1]College of Resources and Environment, Southwest University, Beibei, Chongqing 400715, China. [2]College of Environmental and Resource Sciences, Zhejiang A&F University, Hangzhou 311300, China. [3]Guizhou Provincial Tobacco Company Zunyi Branch, Zunyi 563000, China.

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