Antifungal and Biocontrol Evaluation of Four *Lysobacter* Strains Against Clubroot Disease

Lina Fu¹ · Hanmei Li¹ · Lanfang Wei¹ · Jun Yang¹ · Qi Liu¹ · Yating Wang¹ · Xing Wang¹ · Guanghai Ji¹

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Abstract The effect of crude extract (Ce), seed coating agent (SCA) and whole bacterial broth culture (WBC) of *Lysobacter* strains was evaluated against the causal agent of clubroot formation in Cruciferous vegetables. The ability of four *Lysobacter* strains (*L. antibioticus* 6-B-1, *L. antibioticus* 6-T-4, *L. antibioticus* 13-B-1 and *L. capsici* ZST1-2) inhibited *Plasmodiophora brassicae* of resting spores and disease. Application of WBC of four *Lysobacter* strains inhibited clubroot disease, indicating that the disease suppression was due to antifungal compounds produced by the biocontrol bacterium in the culture. Development of clubroot on Chinese cabbage was inhibited when the WBC and SCA were applied before *P. brassicae* inoculation. Crude extract (Ce) of culture filtrate was effective in arresting the germination of resting spores of *P. brassicae* on slides. However, *Lysobacter* strains differed in their biocontrol effects, the strain *L. capsici* ZST1-2 recorded a high level of disease limiting effect.

Keywords Clubroot disease · *Lysobacter* strains · Resting spores germination · Biocontrol efficacy

Introduction

Clubroot disease of cruciferous vegetables (family Brassicaceae, also called Cruciferae), caused by the obligate biotroph *Plasmodiophora brassicae* Wornmin, is one of the most damaging diseases threatening production in the world. It causes underground root tumor, and aboveground wilt resulting in final death of plants. Presently, the disease occurs in more than 10,000 hectares globally. With farm mechanization and popularization of agro-technologies such as greenhouse seedling and float breeding further accelerate the importance of clubroot disease epidemics [1], especially in Yunnan Province and Sichuan Province in China [2]. Clubroot disease spreads mainly through dispersal of resting spores in soil, farm implements, irrigation water, rainwater and manure [3–5]. It is difficult to control as its pathogen survives in soil for at least 15 years. However, several measures or studies have illustrated the potential for reducing this disease [6], such as application of fungicides in soil to reduce root hair infection or cortical infection, soil pH and nutrient management [7]. These measures destroy the soil structure and the effect of disease control is poor [4, 5, 8]. Besides, pathogen variation giving rise to physiological races of *P. brassicae* is fast, so disease resistance is unstable [4, 9–11]. Biological control of clubroot is attractive because certain soil microbes can colonize roots and/or the rhizosphere [12, 13], producing antifungal metabolites against *P. brassicae*, and by inducing host resistance to the disease [14], thereby can potentially provide durable protection.

Lysobacter spp. occupies an important position and is efficient antagonist of phytopathogens and potential candidates for biological control of crop disease [15] amongst the assessment of potential microbes from the crop rhizosphere regions [16]. Several antagonistic metabolites are

✉ Guanghai Ji
1024422659@qq.com

¹ Key Laboratory of Agriculture Biodiversity for Plant Disease Management Under the Ministry of Education, Yunnan Agricultural University, Kunming 650201, People's Republic of China

produced by *Lysobacter* species, for example, *L. antibioticus* produces a broad spectrum of antibacterial Myxin that inhibits most of bacteria and fungi [17]. *L. antibioticus* degrades the chitin layer of nematodes *Heterodera schachtii* Schmidt and *Heterodera glycines* and eventually kills them [18]. *L. antibioticus* HS124 produces lytic enzymes and a toxic compound against *Phytophthora capsici* [19]. Likewise, the production of antibiotics and lytic enzymes were shown for *L. enzymogenes* OH11 against *Fusarium graminearum*, *Rhizoctonia solani* and *Pythium aphanidermatum* [20, 21]. Furthermore, *L. capsici* AZ78 produces secondary metabolites that inhibit the growth of phytopathogenic oomycetes (*Phytophthora infestans* and *Plasmopara viticola*) [22–24]. However, the past reports mainly focused on antifungal activities, structures, and synthesis mechanism. Few studies have elucidated the antifungal effect and biocontrol of different *Lysobacter* strains on *P. brassicae* Woronin. Biocontrol studies of *Lysobacter* strains on clubroot have not been performed. Indeed, it has been reported that *Lysoabacter* has the potential for biological control of several crop diseases, and has also been observed to inhibit a few phytopathogenic oomycetes in vitro and in vivo [15, 21, 23, 25], but not *P. brassicae*, causing rape clubroot disease.

The current study aimed at investigating the anti-fungal activity of crude extract (Ce), biocontrol effect of seed coating agent (SCA) and whole broth culture (WBC) of four *Lysobacter* strains. We characterized in particular the direct activity of different *Lysobacter* species against spore germination of *P. brassicae* in vitro. Besides, in vivo biocontrol efficacy against the clubroot disease on cruciferace vegetable crops was further evaluated.

Materials and Methods

Chemicals, Reagents and Media

Solid media used in the study consisted of nutrient agar and King's medium B (KB: proteose peptone No. 3, 20 g; glycerin, 10 mL; $K_2HPO_4 \cdot 3H_2O$, 1.5 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; agar, 18 g; distilled water, 1 L; and pH of 7.0).

Hoagland nutritive medium (Mother liquid A: KNO_3 , 101.11 g; KH_2PO_4 , 27.28 g; $MgSO_4 \cdot 7H_2O$, 98.61 g; distilled water, 1 L; Mother liquid B: $Ca(NO_3)_2$, 205.05 g; distilled water, 1 L; Mother liquid C: H_3BO_3 , 32.56 g; $MnCl_2 \cdot 7H_2O$, 1.81 g; $ZnSO_4 \cdot 7H_2O$, 0.22 g; $H_3MoO_4 \cdot H_2O$, 0.09 g; $CuSO_4 \cdot 5H_2O$, 0.05 g; distilled water, 1 L; and Mother liquid D: $FeSO_4 \cdot 7H_2O$ 1.39 g, $Na_2 \cdot EDTA$ 1.86 g, distilled water, 1 L. Mix mother liquid A 4 mL, mother liquid B 5 mL, mother liquid C 1 mL and mother liquid D 4 mL, and make-up the volume to 1 L).

Coating formulations (CA) (4% poval:4% chitosan:2% xanthan gum = 4 V:1 V:1 V).

Seed coating agent (SCA): Cells re-suspended in water (CRW, containing only the bacterial cells, devoid of the culture medium constituents) mixed with a coating agent (CA) at 1:10 proportion designated.

Lysobacter Strains and Culture Conditions

L. capsici ZST1-2 (KR092191, seed epispem of purple potato from Fuyuan County of Yunnan Province, PRC), *L. antibioticus* 13-B-1 (KR092190, seed epispem of *Amorphophallus konjac* from Fengqing County of Yunnan Province, PRC), *L. antibioticus* 6-B-1 (KR092188, seed epispem of *Amorphophallus konjac* from Fengqing County of Yunnan Province, PRC) and *L. antibioticus* 6-T-4 (KR092189, rhizosphere soil of *Amorphophallus konjac* from Fengqing County of Yunnan Province, PRC), stored at length in glycerol 20%, at $-80^\circ C$ was used. These were inoculated on KB agar plate to obtain separated colonies. The primary seed culture was obtained by overnight incubation of a single colony in 20 mL of KB medium with 160 rpm, at $28^\circ C$ overnight, and then second seed culture was harvested from 100 mL of KB medium after overnight growth under same conditions by inoculating suspension of the primary seed culture. The individual *Lysobacter* strain cultures were obtained from growth in 5 L of the same media after 3-day incubation which were derived by inoculating suspension of the second seed culture.

Extraction of Metabolites from *Lysobacter* Strains Culture Filtrates (CFs)

Metabolites from *L. antibioticus* were extracted according to the method described by Zhao et al. [26]. The culture supernatants of *L. antibioticus* strains 13-B-1, 6-B-1 and 6-T-4 were obtained from cultures grown in KB medium as described above by centrifugation ($10,000 \times g$, $4^\circ C$ for 10 min) and designated as culture filtrates (CFs). Then the CFs were extracted with equal volumes of ethyl acetate for three times, and the pooled organic phase was evaporated to dryness under vacuum and dissolved in minimal quantities of 1% MeOH to obtain the crude extract (Ce).

Extraction of metabolites from *L. capsici* ZST1-2 was performed according to the method described by Puopolo et al. [23] with some modifications. The CFs of *L. capsici* ZST1-2 produced in KB medium as described above was centrifuged ($8000 \times g$, $4^\circ C$ for 10 min). Subsequently, CFs (5.0 L) were extracted with equal volumes of ethyl acetate for three times. The organic extracts were combined, dried (Na_2SO_4) and evaporated under reduced pressure to obtain the crude extract.

Extraction and Purification of the Resting Spores of *P. brassicae*

The resting spores of *P. brassicae* were collected according to the method described by Yang et al. [27] with some modifications. Approximately 30 g of *P. brassicae* clubbed Chinese roots frozen at -80°C was homogenized in 150 mL of sterile distilled water (ddH₂O) in a blender, and filtered through 8 layers of cheesecloth. The pellet containing resting spores, was washed with ddH₂O and was recovered by centrifugation ($500\times g$, 4°C , 5 min). The supernatant was discarded and the residue was collected by centrifugation ($4000\times g$, 4°C , 10 min). The residue was re-suspended in 5 mL of 50% sucrose and centrifuged at $2000\times g$ and kept for 10 min at 4°C . The supernatant was collected diluted with 20 mL of sdH₂O and centrifugation at $4000\times g$ and 4°C for 10 min and the residue was resuspended in 30 mL ddH₂O and centrifuged again as above for 10 min. The residue containing the cleaned up purified resting spores was stored at 4°C and used within 48 h.

Inhibition of Spore Germination of *P. brassicae* by the Crude Extract (Ce) of Four *Lysobacter* Strains

The crude extract obtained from the culture filtrates of four *Lysobacter* strains was dissolved in 1% MeOH and used to treat the prepared resting spores in 2 mL microcentrifuge tubes with 700 μL of resting spores and 700 μL of crude extract (final concentration: resting spores $10^8/\text{mL}$, crude extract 500 $\mu\text{g}/\text{mL}$), and 700 μL of resting spores treated by 700 μL 1% MeOH as control. All treatments were incubated in a moist chamber at 28°C in the dark. After 48 h of incubation, each treatment 10 μL was taken on microscope slide and stained with Evan blue for 3 min [28]. The excess stain was removed and the germination of the resting spores were evaluated under fluorescence microscope (Leica Instrument Co. Ltd., Germany and the proportion of germination was derived using the formula) [29]: The treatments were replicated three times. The test was repeated once. Computational formulas (Spore germination rate (%)) = (number of germinated spores/total number of spores) $\times 100$; Spore germination inhibition rate (%) = (control spore germination rate – treatment spore germination rate)/control spore germination rate $\times 100$).

Evaluation of Biocontrol Potential of WBC and SCA of Four *Lysobacter* Strains on Clubroot Diseases

Four *Lysobacter* strains cultured following above described fermentation method yielded ca. 10^9 CFU/mL. This was used as whole bacteria broth culture (WBC). WBC

consisted of bacterial cells suspended in the culture solution. WBC was diluted 5 times and used as the standard treatment (root-irrigated 10 mL 1/5 WBC per plant). Distilled water was used in place of the culture fluid for control. Besides, WBC was separated into cellular and supernatant fractions by centrifuging suspensions at 12,000 rpm for 5 min. The cells were washed three times with distilled water, and re-suspended in sterile distilled water. This preparation was designated as cells re-suspended in water (CRW). This CRW containing only the bacterial cells (ca. 3.0×10^8 CFU/mL, devoid of the culture medium constituents) was mixed with a coating agent at 1:10 proportion designated as SCA (containing the bacterial cells ca. 3.0×10^7 CFU/mL) was used for plant inoculation separately. The Chinese cabbage seeds were washed three times with sterile water followed by soaking the seeds (15 g) in SCA for 3 mL and air dried in the shade. Seeds soaked in CA (3 mL) and air dried in the shade served as the control. SCA and CA treated seeds were planted 20 seeds per pot, separately.

Greenhouse experiments were conducted at Yunnan Agricultural University, Kunming City, Yunnan Province, China, in the summer of 2015 and 2016. All treatments were induced by artificial inoculation of *P. brassicae* spores. Seeds (Chinese cabbage (*Brassica rapa*) cv: Lu Chun Bai No.1, Qingdao International Seed Co. Ltd. China) were sown in plastic pots (90 cm width, 210 cm length) filled with potting mixture (Soil fraction according to sterilized soil: peat: vermiculite: perlite = 1 V:2 V:1 V:1 V). Plants were maintained at $25\text{--}30^{\circ}\text{C}$ and relative humidity between 60 and 70% for 7 weeks. The soil in each pot was kept moist by watering daily. There were three replicates for each treatment and both experiments were performed at sprout after 1 week. WBC treatment, SCA treatment and the control plants were inoculated with *P. brassicae* spores (1×10^8 spores/mL) at 10 mL per plant. Disease index (DI) and control efficacy (CE) were evaluated 50-days after planting according to Sun et al. [16] and DI and CE were derived according to the following formulae.

Disease index (DI), values ranged from 0 to 100, and was calculated for each pot.

$$\text{DI} = \left[\frac{\sum (\text{the number of diseased plants in the pot} \times \text{rating } i)}{(\text{total number of plants in the pot} \times 9)} \right]$$

where i denoting disease intensity is 0–9.

Control Efficacy (CE) is the relative effectiveness of a treatment in controlling clubroot severity expressed on a percentage basis, then was calculated from the mean disease index for that treatment using the equation:

$$CE = \left[\frac{(\text{mean DI of the control} - \text{mean DI of the treatment})}{\text{mean DI of the control}} \right] \times 100\%$$

Data Analysis

Analysis of variance was performed on data from individual experiments using SPSS 19.0. The experimental data from inhibition of spore germination were subjected to factorial analysis of variance with crude extract of four *Lysobacter* strains as the factors. Results from repeated pot experiments were pooled in the statistical analysis after error variance were found to be homogeneous. Mean comparisons were performed using Duncan test ($P = 0.05$).

Results

Evaluation of Crude Extract (Ce) from *Lysobacter* Strains Against *P. brassicae*

Plasmodiophora brassicae resting spore germination was significantly inhibited by Ce of all the four *Lysobacter* strains Table 1. Among the stains the inhibition rates of Ce from *L. antibioticus* 6-B-1, *L. antibioticus* 6-T-1 and *L. capsici* ZST1-2 higher than in *L. antibioticus* 13-B-1.

Microscopic Observation of Spore Germinate

Plasmodiophora brassicae resting spores upon germination become empty, while the non-germinated ones did not loose the cell contents (Fig. 1).

Effect of SCA on Club Root Inhibition

Incidence of clubroot was significantly ($P < 0.05$) inhibited by the bacterial cell preparations (SCA) of *Lysobacter* strains (Table 2). The best control effect on clubroot was obtained with *L. capsici* ZST1-2.

Effect of WBC on Club Root Inhibition

Similar to the effect of SCA, the whole broth culture (WBC) inoculum also significantly inhibited club root formation (Table 3). Moreover, root swelling of *P. brassicae* was alleviated the most by treated with WBC of *L. capsici* ZST1-2 compared to control (Fig. 2). Interestingly, the disease control was higher in this compared to that of SCA.

Discussion

Bacteria belonging to the *Lysobacter* genus are frequently found in soil and their effect on suppressing soil borne pathogens has been established [30, 31]. Species of *L. antibioticus* and *L. capsici* are being extensively used as beneficial rhizobacteria for biocontrol of plant disease owing to their ability to produce a broad array of bioactive metabolites, among which antibiotic and lytic enzymes, have been well-recognized [19, 32, 33]. Through combination of the inhibition of resting spores test and control efficacy experiments in greenhouse, we were successful in selecting *L. capsici* ZST1-2 as the efficient biological control agent, especially when the whole broth culture (WBC) was used. This was supported by the in vitro tests on spore germination suggesting that factors such as antibiotic substances and enzymes from bacterial metabolism might have contributed to the inhibitory effect. Nevertheless, the in vitro experiment revealed that *Lysobacter* strains had effective in controlling clubroot disease under greenhouse conditions. The greenhouse experiment, despite the affect environmental factor of its environment was single than in the field, is an effective tool for selecting candidate organisms for evaluating their biocontrol efficacy. These results showed that *Lysobacter* genus as an important and potential biocontrol agent in controlling clubroot disease.

In recent years, some biocontrol strains have been reported to control clubroot disease of Cruciferous plants, such as *Streptomyces* spp., *Trichoderma* spp., and *Bacillus*

Table 1 Effects of clubroot resting spores germinated treatments with Ce from four *Lysobacter* strains

<i>Lysobacter</i> strains	The germination rate of resting spore/%	The germination inhibition rate of resting spore/%
ZST1-2	29.67 ± 2.9734c	67.98 ± 1.6286a
13-B-1	43.67 ± 1.8366b	52.88 ± 2.3161b
6-B-1	33.67 ± 0.6518c	63.67 ± 1.1262a
6-T-4	35.00 ± 0.6807c	62.23 ± 1.5352a
sdH ₂ O (control)	92.67 ± 1.4904a	–
<i>P</i>	< 0.05	< 0.05

Means in a column followed by the same letter are not significantly different at $P = 0.05$ based on Duncan test. The results is resting spores were treated for 24 h

Fig. 1 Evan blue staining of *Plasmodiophora brassicae* spores treated with Ce of *L. capsici* ZST1-2. **a** Sterile distilled water, and arrowhead **a** indicates germinated spores. **b** Ce of *L. capsici* ZST1-2, staining for 15–20 s after spores have been treated for 24 h. And

arrowhead **b** indicates germinated spores, arrowhead **c** indicates non-germinated spores. **c** Ce of *L. capsici* ZST1-2, staining for 15–20 s after spores have been treated for 48 h. Arrowhead **d** indicates non-germinated spores

Table 2 Clubroot disease treated with SCA of four *Lysobacter* strains under greenhouse conditions

Strains	Incidence/%	DI (0–100)	CE/%
6-B-1	73.6 ± 2.0640ab	18.71 ± 0.8920cd	46.68 ± 1.4290b
6-T-4	77.7 ± 5.8682ab	21.60 ± 0.5369bc	38.44 ± 1.7301c
13-B-1	78.9 ± 2.5541ab	22.8 ± 0.1531b	35.00 ± 0.3055c
ZST1-2	66.67 ± 4.4715b	16.93 ± 0.8426d	51.75 ± 0.9412a
No treatment	84.21 ± 2.3814a	35.0 ± 1.8240a	–
<i>P</i>	< 0.05	< 0.05	< 0.05

Means in a column followed by the same letter are not significantly different at $P = 0.05$ based on Duncan test

Table 3 Clubroot disease treated with WBC of four *Lysobacter* strains under greenhouse conditions

Strains	Incidence/%	DI (0–100)	CE/%
6-B-1	73.08 ± 0.8100c	20.09 ± 1.1642c	53.93 ± 1.9096a
6-T-4	78.95 ± 2.3750b	20.47 ± 1.700c	53.06 ± 1.1175a
13-B-1	82.14 ± 1.5480b	24.21 ± 0.8574b	44.49 ± 1.3767b
ZST1-2	53.33 ± 0.8641d	20.00 ± 0.1323c	54.14 ± 1.600a
No treatment	92.50 ± 0.8329a	43.61 ± 0.4949a	–
<i>P</i>	< 0.05	< 0.05	< 0.05

Means in a column followed by the same letter are not significantly different at $P = 0.05$ based on Duncan test

Fig. 2 Inhibition of clubroot disease treated with WBC of *L. capsici* ZST1-2 before inoculation with *Plasmodiophora brassicae*. **a**: no treatment (control). **b** treated with WBC of *L. capsici* ZST1-2 before inoculation with *P. brassicae*, root swelling was markedly alleviated

subtilis XF-1 [34, 35]. *L. antibioticus* has been reported as a new type of biocontrol bacteria for controlling clubroot disease of Chinese Cabbage and this bacterium showed strong antagonism [36]. This is the first time to use crude extract, seed coating agent and whole bacteria broth culture of four *Lysobacter* strains to demonstrate inhibition of spore germination in the fungal pathogen combined with control effect of greenhouse-grown plants and to estimate the efficacy of *Lysobacter* strains to control clubroot disease. Biocontrol mechanism of *L. antibioticus* might be through its secondary metabolites as 1,6-dimethoxy phenazine and butyl phthalates, produced by *Lysobacter* has been shown to inhibit the germination of resting spores [37]. Further *L. capsici* produces cyclo (L-Pro-L-Tyr), a 2,5-diketopiperazine toxic to sporangia of plant pathogenic oomycetes [23]. However, it is not clear whether the mechanisms of antifungal activity differs between different *Lysobacter* strains, they are considered as good candidates for the development of new eco-friendly products that can be applied for a more sustainable control of plant pathogenic oomycetes. Besides, chitosan in SCA was able to reduce clubroot, inhibit spore germination and clubroot occurrence, this may increase the antagonism of biocontrol strains against spores [38, 39]. Nevertheless, the SCA treatments were lower inefficient compared to WBC in this study perhaps due to the fact that SCA consists of cells resuspended in water (CRW), and only use small fraction of viable bacterial cells, on the other hand, WBC treatments use composite components including Ce from CFs and CRW, its inhibitory effect on *P. brassicae* spores and clubroot formation was more effective. Whether these bacterial agents could penetrate roots and stimulate endophytes to strengthen clubroot resistance potential of the host plant needs further study [14]. However, this study reveals the utility of *L. capsici* ZST1-2 for use as a biocontrol agent against clubroot formation caused by *Plasmodiophora brassicae* in Cruciferous vegetables.

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Compliance with Ethical Standards

Conflict of interest The authors declares that they have no conflict of interest.

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