Role of antagonistic *Lysobacter* species and their bioactive compounds in suppression of plant diseases

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Content

Abstract

Rhizoctonia solani is a fungus that causes soil-borne diseases and major yield losses in multiple crops worldwide. *Lysobacter* spp. isolated from *Rhizoctonia* suppressive soils might play a role in the disease suppression of *R. solani*. *Lysobacter* spp. are able to inhibit *R. solani in vitro* but a detailed understanding of the mechanisms is still lacking. In this study I will elucidate the role of chitinases produced by *Lysobacter antibioticus* strain L08, *L. capsici* strain L14, *L. gummosus* strain L15 and *L. enzymogenes* strains L19 and L29 in suppression of *R. solani*. To that end, activity assays were performed to test whether the *Lysobacter* species can inhibit *R. solani in vitro*. A bioassay in which the *Lysobacter* species were added to the conducive soil and cauliflower plants were sown showed no consistent *R. solani* disease suppression. Rhizosphere colonization showed that the colonization for the *Lysobacter* species was sufficient enough for disease suppression, except for *Lysobacter capsici* where it remains unclear if it can colonize. All five *Lysobacter* strains showed chitinase activity *in vitro*. qPCR showed that the *chiA* gene, encoding for chitinase A, was downregulated for *Lysobacter enzymogenes* in the presence of *R. solani* cell material. The other *Lysobacter* species did not have down- or upregulation of *chiA* in the presence of *R. solani* cell material. *In vitro* activity of all strains against *R. solani* was lowered when chitin was added to the medium in comparison to medium without chitin. HPLC analysis has identified the compounds that are present and absent when *Lysobacter* species were grown on medium with and without chitin. To obtain an in frame deletion of the *chiA* gene, site-directed mutagenesis via the pEX18Tc vector has been performed, but the final transformation step was not successful. After this study the precise role of *Lysobacter* species on disease suppression of *R. solani* in the soil remains absent, but chitinases do seem to affect the production of antifungal compounds.

Introduction

Rhizoctonia solani is a basidiomycete fungus that causes soil-borne diseases on numerous plant hosts, including wheat, sugar beet, radish, rice, potato and cauliflower. Several of these plant species are of significant economic value due to their large scale cropping (Postma *et al.*, 2010, Gonzalez *et al.*, 2011). To date, chemical and genetic measures to control this pathogen have not been effective. Therefore, there is an increased interest in microorganisms that have activity against *R. solani* and which could be developed into biocontrol agents. From several agricultural soils, several *Lysobacter* spp. were isolated (Postma *et al.*, 2010, Postma *et al.*, 2008) and proposed as potential candidates for biocontrol of *R. solani* and other plant diseases.

Rhizoctonia solani

R. solani is a soil-borne fungus that can survive without a host plant as a sclerotium or as a saprophyte (Fig. 1). Infection occurs through hyphae that have emerged from basidiospores under wet conditions or directly by mycelium. Hyphae are attracted by exudates released from the roots of the host plant. When the hyphae come into contact with the host, they grow over the surface and the infection process can start. The round-shaped hyphae become flattened and start making T-shaped branches. These T-shaped branches can give rise to short swollen hyphae or appressoria-like structures or they form repetitive T-shaped branches. In extreme forms the infection structures can be seen as infection cushions. Infection pegs are then formed from swollen hyphal tips that can penetrate the cuticle and epidermal cell wall. *R. solani* often enters through intact tissue although it can also enter through wounded tissue, lenticels and stomata (Sneh *et al.*, 1996).

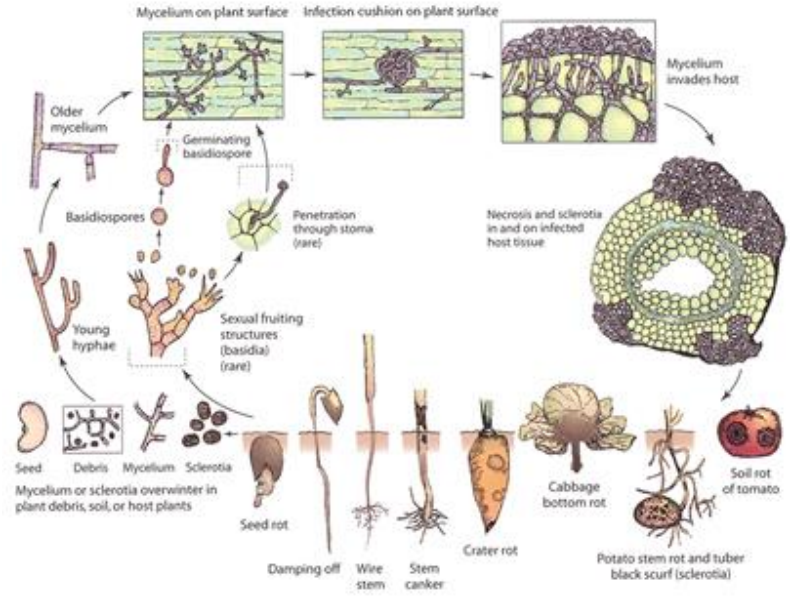

Figure 1. Disease cycle of *R. solani* (Agrios, 2005)

R. solani isolates can be grouped into anastomosis groups (Postma *et al.*), which are traditionally identified by hyphal anastomosis reactions. Anastomosis is the fusion or merger of hyphae between isolates of the same AG. So far 13 AGs are known for *R. solani* (Carling *et al.*, 2002). *R. solani* spp. belonging to different anastomosis groups often have different host plants and can differ in the type of disease symptoms (Pannecoucque & Hofte, 2009). The most common disease symptom is damping-off, the killing of the plant before or after germination (Gonzalez *et al.*, 2011). In older plants, lower stem- and root rot (called sore shin) occurs. Lesions on the stem turn from brown to black. These symptoms often occur in sugar beet and are caused by *R. solani* AG-1 , AG 2-2 and AG-4 (Gonzalez *et al.*, 2011).

Lysobacter species and their bioactive compounds

An agricultural soil in Zwaagdijk (the Netherlands), where cauliflower had been successively grown, was found to be suppressive against *R. solani* (Postma et al., 2008). In this soil, *Lysobacter* isolates were more abundant compared to a nearby located pear orchard soil that is conducive to *R. solani* (Postma *et al.*, 2010), even though the two soils have similar physical and chemical properties and both contained *R. solani* (Postma *et al.*, 2010). The suppressive soil contained five to twelve times more *Lysobacter* than the conducive soil (Postma *et al.*, 2010). The isolated *Lysobacter* species from this suppressive soil were *Lysobacter antibioticus*, *Lysobacter capsici*, and *Lysobacter gummosus* (Postma *et al.*, 2011).

Lysobacter spp. are potential biological control agents (Hayward *et al.*, 2010, Postma *et al.*, 2011), since they have activity against several bacterial pathogens, including *Xanthomonas oryzae* on rice, as well as oomycete pathogens on pepper, sugar beet and cucumber (Table1). *Lysobacter* species produce several antimicrobial metabolites (Table 2). For example, *Lysobacter enzymogenes* produces HSAF (Heat Stable Antifunal Factor) that can inhibit fungi (Folman *et al.*, 2003). *Lysobacter gummosus* is able to produce 2,4-diacetylphloroglucinol(DAPG)(Hashizume *et al.*, 2011). In general all *Lysobacter* species produces β- 1,3- glucanases and chitinases that are able to inhibit fungi. For *Lysobacter antibioticus and Lysobacter* capsici is not much known.

Table 1. an overiew of pathogens *Lysobacter* species inhibit.

Lysobacter spp	Disease	Plant	Pathogen	References
Lysobacter sp. SB-K88	Damping-off	sugar beet	*Aphanomyces cochloides*	Islam et al, 2010, Islam et al, 2005
			Pythium sp.	Nakayama et al, 1999
	Damping-off	spinach	*Aphanomyces cochloides*	Islam et al, 2010, Islam et al, 2005
L. antibioticus	Bacterial leaf blight	rice	*Xanthomonas oryzae pv oryzae*	Ji et al, 2008
	Phytophthora blight	pepper	*Phytophthora capsici*	Ko et al, 2009
L. capsici	damping-off, root rot	tomato	*Rhizoctonia solani*	Puopolo et al, 2010
			F. oxysporum f. sp. *lycopersici*	Puopolo et al, 2010
			F. oxysporum f. sp. radicis-*lycopersici*	Puopolo et al, 2010
L. enzymogenes	Damping-off	cucumber	*Pythium aphanideratum*	Folman et al, 2004; Postma et al, 2009
	Damping-off	sugar beet	*Pythium aphanideratum*	Palumbo et al, 2005
	Head blight	wheat	*Fusarium graminearum*	Jochum et al, 2006
	Brown spot	tall fescue	*Rhizoctonia solani*	Giesler and Yuen, 1998
	Leaf spot	tall fescue	*Bipolaris sorokiniana*	Zhang an Yuen, 1999, 2000; Kilic-Ekici and Yuen 2003, 2004; Kobayashi et al, 2005
	Summer patch disease	Kentucky bluegrass	*Magnaporthe poae*	Sullivan et al, 2003; Kobayashi and Yuen, 2005
	Rust	bean	*Uromyces appeniculatus*	Yuen et al, 2001
	Phytophthora blight	pepper	*Phytophthora capsici*	Kim et al, 2008

Chitinases

Chitin is an insoluble polymer of β-1,4-linked N-acetylglucosamine (GlcNAc) present in many insect and fungal cell walls. Chitinases are glycosyl hydrolases that catalyze the hydrolytic degradation of chitin. They are divided into two families of glycosyl hydrolases 18 and 19, based on similarity of the catalytic domain (Song *et al.*, 2013). Family 18 chitinases are present in bacteria, fungi, viruses, animals and some plants (Ningaraju, 2006). They have a common $(\beta/\alpha)_8$ barrel catalytic domain (Perrakis *et al.*, 1994) that first cleaves the sugar chain (N-acetyl) followed by further hydrolysis (van Aalten *et al.*, 2001). Chitinolytic enzymes can be divided into three categories: exochitinases, endochitinases and β-N-acetylglucosaminidase. Exochitinases have activity only at the non-reducing end of the chitin chain and cleave off $(GlcNac)_2$. Endochitinases can hydrolyze internal β-1,4-glycoside and cleave randomly in the chitin chain, resulting in short GlcNac oligomers. β-N-acetylglucosaminidase cleaves GlcNAc units sequentially from the non-reducing end of the substrate (Bhattacharya *et al.*, 2007).

Table 2. an overiew of inhibitory metabolites produced by *Lysobacter* species

Species	Compound	Activity	Gene	References
L. antibioticus	1-hydroxy-6-methoxyphenazine	antioomycete		Cook et al, 1971;
	4-hydroxyphenylacetic acid	antioomycete		Ko et al, 2009
L. enzymogenes	dihydromaltophilin (HSAF)	antifungal/antioomycete	PKS/NRPS	Yu et al, 2007; Li et al, 2009, Lou et al, 2012
	biosurfactant	antifungal/antioomycete		Follman 2003
	cyclic lipodepsipeptide (WAP-8294A2)	anti-MRSA	NRPS	Zhang et al, 2011
L. gummosus	2,4-diacetylphloroglucinol	antifungal		Brucker et al, 2008
L. lactamgenus	cephabacin	unknown		Demilev et al, 2006; Lee et al, 2008
Lysobacter sp.	tripropeptin C (cyclic lipodepsipeptide)	antibacterial		Hashizume et al, 2011
	lysobactin (macrocyclic depsipeptide)	antibacterial	NRPS	Hou et al, 2011
	xanthobaccin A, B & C	antibacterial		Nakayami et al, 1999; Hashizume et al, 2008
	endopeptidase L1,4,5	antibacterial - lysis		Tsfasman et al, 2007; Vasilyeva et al, 2008
	N-acetylmuramoyl-L-alanine amidase	antibacterial - lysis		Tsfasman et al, 2007; Vasilyeva et al, 2008
	muramidase	antibacterial - lysis		Tsfasman et al, 2007; Vasilyeva et al, 2008
Lysobacter spp.	B-1,3-glucanases			Palumbo et al, 2005
	chitinases	antifungal		Zhang et al, 2001; Ko et al, 2009

Bacteria that produce chitinases often have more than one chitinase gene (Horn *et al.*, 2006). *Serratia marcescens* contains up to five different chitinases with each a different protein size ranging from 20 kD to 60 kD (Fuchs *et al.*, 1986). Cloning and subsequent sequencing of the genes encoding these enzymes have resulted in the identification of the *chiA*, *chiB*, *chiC* and *chiD* genes, each encoding for an enzyme with a different protein size (Watanabe *et al.*, 1990). The chitinases share the catalytic domain, but they possess one or more smaller subdomains thought to be involved in specific substrate binding (Horn *et al.*, 2006). *chiA*, *chiB* and *chiC* are the best characterized enzymes. *chiA* has a N-terminal substrate binding domain. *chiB* has a linker and a C-terminal chitin binding domain. *chiC* only has a C-terminal chitin binding domains. It is thought that despite having catalytic domains with similar folds, *chiA* functions as an endochitinase and *chiB* as an exochitinase, degrading chitin from the opposite ends. *chiC* has a lower catalytic efficiency for short substrates than *chiA* and *chiB* (Horn *et al.*, 2006).

Chitinase production is influenced by several biotic and abiotic factors. For example, chitinases are often upregulated by the presense of chitin or chitin derivatives like colloidal chitin. Also, nutrient rich medium repressed chitinase production, which was mainly attributed to high glucose concentrations (Bhattacharya *et al.*, 2007, Folders *et al.*, 2001). Other factors that influence chitinase activity are pH and temperature. The optimal pH for chitinases can vary from 4.5 to 10. Different optimal temperatures have been found for *Streptomyces violaceusniger* (28°C) and *Streptomyces thermoviolaceus* (80°C) (Bhattacharya *et al.*, 2007). Little is known about the genetic regulation of the chitinase genes. An overexpressing chitinase mutant was identified in *S. marcescens*, but the mutated gene has not been identified so far (Reid & Ogrydziak, 1981). In *L. enzymogenes*, a mutation in genes encoding for the glucose/galactose transporter (*gluP*), S-adenosylmethionine decarboxylase (*speD*) and a disulphide bond formation protein B (*dspB*) abolished chitinase production in *L. enzymogenes* C3, indicating a role in the regulation of chitinase production (Choi *et al.*, 2012). Also, a mutation in catabolite activator protein-like (*clp*), a known global regulator for lytic enzymes, gliding motility and *in vitro* antimicrobial activity, reduces chitinase activity of *L. enzymogenes* (Sullivan *et al.*, 2003, Choi *et al.*, 2012). Whether these genes play a role in chitinase production in other *Lysobacter* spp. is not known. With regards to secretion of chitinase, also not much is known. *chiA* in *Vibrio cholerae* is secreted into the cellular medium via the type II secretion pathway. In *Pseudomonas aeruginosa* it is postulated that *chiC* is secreted by a novel secretory pathway (Folders *et al.*, 2001).

Since *Lysobacter* spp. are known to produce chitinases (Hayward et al., 2010) and most fungal cell walls have chitin as major building brick these chitinases might contribute to antifungal activity (De Boer et al., 2001). A tobacco plant over expressing plant chitinases has been found more resistant against *R. solani* (Gonzalez et al., 2011). Also, chitinases produced by bacteria are inhibiting *R. solani* growth, as was shown for *S. marcesens* (Song et al., 2013). In *Lysobacter enzymogenes* the gene encoding for chitinase activity, *chiA*, was either mutated or deleted and tested for antifungal activity

(Qian *et al.*, 2012). Besides a loss in chitinase activity on chitin medium, the antifungal inhibition was not lost, indicating that chitinases were not required for antifungal activity. However, this is not known for the chitinases produced by other *Lysobacter* spp.

Research question

Do *Lysobacter* species play a role of disease suppression of *R. solani* in cauliflower and are chitinases involved?

Material and methods

Strains and culture conditions

Lysobacter strains L08 (*L. antibioticus* L08), L14 (*L. capsici* L14), L15 (*L. gummosus* L15), L19 (*L. enzymogenes* L19), L29 (*L. enzymogenes* L29) were maintained on R2A medium (Difco) and grown at 25 °C for 3 days. When cultured in liquid medium, Luria Burti's or R2B medium were used. *R. solani* AG2-1 and AG2-2IIIB were grown on 1/5 PDA medium at 25°C.

In vitro activity against *R. solani* AG2-2IIIB and AG2-1 on R2A

All *Lysobacter* strains were grown in 10 ml LB for three days at 25°C at 200 rpm. Suspensions were centrifuged at 4643 g for 10 minutes and cells were washed three times and resuspended in 10 ml 0.9% NaCl. The cell density was measured with a spectrophotometer at a wavelength of 600 nm, and subsequently diluted to the required density in 0.9% NaCl. 50 µl of 10^9 cells/ml was spot inoculated on R2A medium. After overnight incubation at 25°C a plug,with a diameter of 5mm of *R. solani* AG2-1 or AG2-2IIIB was placed in the middle. The plates were incubated for 5 days at 25°C and photographed. To test the presence of chitin on inhibition, R2A was supplemented with 0.2% or 0.5% colloidal chitin. Also culture filtrates of the *Lysobacter* strains were tested for activity. *Lysobacter* strains were grown in 10 ml R2B, with and without 0.2% colloidal chitin, for three days at 25°C at 200 rpm and centrifuged 4643g for 10 minutes. The supernatant was filter sterilized with a 0.2 µm filter (Whatman). 50 µl of the supernatant was spot inoculated on R2A medium, R2A medium supplemented with 0.2% or 0.5% colloidal chitin. A plug of *R. solani* AG2-1, grown on 1/5 PDA, was placed in the middle of the petridish. After 5 days incubation at 25°C pictures were taken.

In vivo bioassay

rifampicin resistant mutants of the *Lysobacter* strains were grown in 10 ml LB, supplemented with 100 µg/ml rifampicin for three days at 25°C at 200 rpm. Suspensions were centrifuged at 4643 g for 10 minutes and cells were washed three times and resuspended in 10 ml 0.9% NaCl. The cell density was measured with a spectrophotometer at a wavelength of 600 nm, and subsequently diluted to the required density in H_20. The treatments consist of L08, L14, L15, L19, L29 and a combination of L08, L14 and L15 in a 1:1:1 ratio with 10^5 and 10^7 cells/g soil. Cell suspensions were mixed through the conducive Zwaagdijk soil with a final water content of 20%. The soil was divided into eight containers (replicates), each containing 250 g soil. The next day, cauliflower seeds (L2012-00003) were sown. A trial has been performed to test for type of container and amount of seeds sown (Fig. 2 and Appendix 1). Plant growth and disease scoring was most optimal when 16 seeds were sown in a rectangle-shaped container (20 mm by 7 mm by 2.5 mm, right panel Fig. 2), so this set-up was used for further bioassays. Plants were grown in a climate chamber at 24 C, 70% humidity with 16 h of light and 8 h of dark.

Figure 2. Left panel: square pot containing 9 cauliflower plants. Right panel: rectangular container containing 12 cauliflower plants. On the right side of the container *R. solani* was inoculated and two plants suffer from *R. solani* disease symptoms.

R. solani AG2-1 was grown for 5 days grown on 1/5 PDA and a plug of 5 mm diameter was placed into the soil touching the first plant seven days after sowing the seeds. Germination percentage was scored at day 11. During a 15-day period after inoculation of *R. solani* disease incidence and distance was scored every two to three days. After 15 days the bioassay was finalized and rhizosphere samples had been taken.

Colonization

Cauliflower rhizospheres were collected from healthy plants that were the closest to the infected seedlings. Per replicate 2-4 plants were taken depending on how many uninfected plants there were left. Two replicates were pooled. Rhizospheres were resuspended into 4 ml 0.9% NaCl and vortexed for 1 minute, sonicated for 15 seconds and vortexed again for 15 seconds. 50µl of a 10x, 100x and 1000x time dilution was plated on selective medium, R2A supplemented with 50 µg/ml rifampicin, 200 µg/ml ampicillin, 25 µg/ml kanamycin and 100 µg/ml Delvocid. Plates were incubated for maximum of 7 days at 25°C. Colony forming units (cfu) were counted once they appeared on plate and cfu/g rhizosphere was calculated.

BOX-PCR

The colonies obtained with the colonization assay were analyzed by BOX-PCR. A colony is picked with a toothpick and resuspended in 50 µl miliQ water. A BOX-PCR reaction of 25 µL is composed of 5 µL cell suspension, 1 µL BOX-A1R primer(10 µM), 1.25 µL dNTPs(100 mM each) 0.4 µL BSA(10 mg/ml), 2.5 µL 100% DMSO, 5 µL 5x Gitschier buffer, 0.4 µL Taq polymerase, 5U µL (SuperTaq) and 9.45 µL miliQ water. The 25 µL is heated up to 95°C for 2 min, then 34 times a cycle of 3 seconds at 94°C followed by 92°C for 30 seconds, 50°C for 1 minute and 65°C for 8 minutes. After 30 cycles samples were incubated at 65°C for 8 minutes and then kept at 8°C. 5 µL of the PCR product was loaded on an 1.5% agarose gel and ran for 8 hours at 40 V or overnight at 20V.

Chitinase activity

All *Lysobacter* strains were grown in 10 ml LB for three days at 25°C at 200 rpm. Suspensions were centrifuged at 4643 g for 10 minutes and cells were washed three times and resuspended in 10 ml 0.9% NaCl. The cell density was measured with a spectrophotometer at a wavelength of 600 nm, and subsequently diluted to the required density in 0.9% NaCl. 50 µl of 10^9 cells/ml was spot inoculated on R2A medium, R2A medium supplemented with 0.2% colloidal chitin and R2A medium supplemented with 0.5% colloidal chitin. After 5 days of incubation at 25 °C pictures were taken.

Genome walking

To obtain the complete *chiA* sequence of *L. antibioticus* L08 and *L. gummosus* L15, a PCR was performed with forward primer 5'-CAA CGT GAT GAC CTA CGA CTT C-3' and reverse primer 5'-GAA GTC GTA GGT CAT CAC GTT G-3'. The PCR product was purified from gel with Nucleospin cleanup kit (Machery Nagel) according to the manufactures protocol and sent for sequencing at Macrogen.

Site-directed mutagenesis

Site-directed mutagenesis was performed with the pEX18Tc vector according to the protocol (Choi & Schweizer, 2005). To amplify the fragment for site-directed mutagenesis of *chiA* in *L. capsici* L14, flanking fragments of the *chiA* gene have been amplified with the following primers: upper fragment forward: 5'-TCA TGC ATG CAA GCT TCG TGC TCA CTT ATG TCG AGG AC-3'; upper reverse fragment 5'-CAT GGT GCT TCC TCT CTC C-3'; down forward fragment: 5'-GGA GAG AGG AAG CAC CAT G AAG TAA TCG GCC TGA CGT G-3'; and down reverse fragment: 5'-AGA TTG ACT CGG TAC CCAT CAC ATA CAG CGT GTC GAG-3'. The PCR-program consist of 3 min at 95°C, 35 times a cycle of 1 min at 95°C, 1 min at 56°C and 1 min at 72°C and it was finished with 5 minutes at 72°C. The upper forward primer contains a *Hind*III restriction site, the lower reverse primer contains a *Kpn*I restriction site for efficient cloning into pEX18Tc. The upper and down fragments were connected via an overlap PCR with the upper forward primer and the lower reverse primer. The PCR program consist of 3 min at 95°C, 35 times a cycle of 1 min at 95°C, 1 min at 56°C and 1 min at 72°C it is finished with 5 minutes at 72°C. This product was ligated into the pGEM-t easy vector

(Promega), according to manufacturer's instructions. The fragment was cut out with the *Kpn*I and *Hind*III restriction enzymes and subsequently subcloned into pEX18Tc, digested with the same restriction enzymes. After overnight ligation at 16°C, the plasmid was transformed into *Escherichia coli* DH5α heat shock competent cells by a heat shock of 30 sec at 42°C, recovery in SOC medium shaking at 200 rpm at 37 °C for 1 h and subsequent plating on LB medium supplemented with 50 µg/ml tetracycline. Plates were incubated at 37°C for 3 days. Correct ligation was confirmed by colony PCR using pEXP18 forward: 5'-CCT CTT CGC TAT TAC GCC AG-3' and pEXP18 Reverse: 5'-GTT GTG TGG AAT TGT GAG CG-3' primer. The PCR program consist of 3 min at 95°C, 35 times a cycle of 1 min at 95°C, 1 min at 56°C and 1 min at 72°C and it was finished with 5 minutes at 72°C. Digestion of isolated plasmid DNA with *Kpn*I and *Hind*III restriction enzymes and loading the samples on a 1% agarose gel. The fragment was purified from gel with the Nucleospin clean-up kit (Machery Nagel) according to the manufacturer's protocol. The isolated fragment was sent for sequencing at Macrogen. Subsequently, the correct pEX18Tc-*chiA* plasmid was transformed into the *Lysobacter capsici* L14 strain via several electroporation or triparental mating. For electroporation, competent cells were generated by a sucrose wash, 2 times washing in 4 ml 300 mM sucrose and resuspending it in 100 µl 300 mM sucrose and 50 ng plasmids was introduced by electroporation using ec2 settings (2.5 kV in a 0.2 cm cuvette, Biorad) settings. Subsequently, cells were plated on selective medium, consisting of either LB or R2A with 100 µg/ml rifampicin and 50 µg/ml tetracycline and incubated at 25°C or 30°C. Cells that were able to grow were then plated on LB medium supplemented with 5% sucrose. A glycerol wash was also performed. rifampicin resistant *L. capsici* L14 were grown in LB medium without NaCl and supplemented with 100 µg/ml rifampicin. After the cell were grown to a density between $0.6*10^8$ and $0.9*10^8$ the cells were spinned down at 1892 g. The medium was removed and the cells were resuspended in ice cold dH_2O. The cells were washed twice in ice cold 10% glycerol and aliquoted in 4 samples Plasmids have been introduced via electroporation as described above.

Plasmid isolation

E. coli DH5α cells were grown on LB supplemented with tetracycline and grown overnight at 37°C at 200 rpm. 1.5 ml of the overnight culture was spinned down at 15870 g for 2 minutes. The supernatant was removed and 200 µl of a solution consisting of 50 mM glucose, 25 mM Tris and 10 mM EDTA adjusted to pH 8.0, was added and mixed by inverting. 200 µl of a solution consisting of 0.2 N NaOH and 1% SDS was added and gently mixed by inverting. 200 µl solution consisting of 60 µl 5 M KAc, 11.5 µl HAc and 28.5 µl H2O was added and gently mixed by inverting. The mixture was spinned for 10 minutes at 15870 g. The supernatant is collected and precipitated with 1 volume isopropanol, spinned for 10 minutes at 15870 g and washed with 70% cold ethanol. The pellet was dissolved in 10 mM Tris with 100 ug/ml RNase.

QPCR

All *Lysobacter* strains were grown and washed as described above. Each treatment was performed with 4 replicates. A starting concentration of 10^6 cells/ml were inoculated in a 24-wells plate (Nunc), each well containing 1.25 ml R2B and R2B supplemented with 33,8 mg/ml dry *R. solani* AG 2-1 cell material, obtained by 5 days incubation of a *R. solani* plug in 25 ml 1/5 PDB. The *R. solani* material was autoclaved and dried overnight at 60°C. The material was grinded and dissolved in R2B. To explore which was the best time point for determining differences in chitinase expression, RT-PCR was perfomed (Appendix 2 and Appendix 3). During the exponential growth phase, the phase between 24 and 48 post inoculation, the expression of *chiA* is the highest. Therefore isolation of RNA is done after 24 hours. Plates were incubated for 24 hours at 25°C at 200 rpm and 1 ml of each culture was centrifuged for 5 min at 20238 g. The cell pellet was stored at -20°C. RNA was isolated from the cell pellet with Trizol (Invitrogen) according to manufacturer's instructions. RNA samples were DNase treated with TURBO DNA-*free*™ (Ambion) and cDNA was made from 1 ug RNA with MMLV reverse transcriptase (Promega) according to manufacturer's instructions. The qPCR mixture consist of 5 µl of the 5 times diluted cDNA, 12.5 µl Sybr green Sensimix (Bioline), 5.5 µl miliQ and 1 µl forward primer and 1 µl reverse primer. The analysis was conducted in 7300SDS system from Applied Biosystems. The program consisted of 2 min at 50°C, followed by 10 min at 95 °C and followed by 40 cycles of 15 sec at 95 °C and 1 min 60°C. The program was finished with a dissociation curve.

Primers for the *rpoD* housekeeping gene have been used as a housekeeping gene for correction of the fluctuations in cDNA concentration between individual samples. The qPCR has been performed with the *chiA* primers: Forward 5'-GCA GCT CAA GGC CAAG C-3'; Reverse 5'-GGT TGC CCT TGA TGT AGG C-3' and *rpoD* primers: Forward: 5'-GTC CGA TAT CAA GCT CCT G-3' and reverse: 5'-CAT GCC GAT GAT GTC TTC G-3'.

HPLC

All *Lysobacter* strains were grown and washed as described above. $2*10^{10}$ cells were dissolved in 200 µl 0.9% NaCl. The cell suspensions were inoculated on R2A, R2A with 0.2% chitin and R2A with 0.5% chitin in a line with a small inoculation loop at one side of the petridish. After overnight incubation at 25°C a plug of *R. solani* AG2-1 grown on 1/5 PDA is placed on the other side of the petridish (Fig. 3). After 5 days of incubation at 25°C the inhibition zone was cut into pieces and dissolved in sterile dH_2O. 6 replicate plates were pooled into one sample. After vortexing for 2 minutes the samples were spinned down for 30 min at 4643 g at 4°C. Trifluoroacetic acid is added to a final concentration of 0.1% and then two volumes of ethyl acetate were added. After overnight incubation at -20°C the ethyl acetate fraction is dried under continuous air flow. The dried extract was dissolved in 1 ml MeOH. A Reverse phase C18 column (5µm), in-line Degasser, 600S Controller, 717 plus Autosampler and 996 Photodiode array Detector have been used for the HPLC analysis. The running solution changed from MilliQ + 0.1% TFA to 100% acetonitrile in 45 minutes, and then back again in 15 minutes, with a flowrate of 0.5 ml/min.

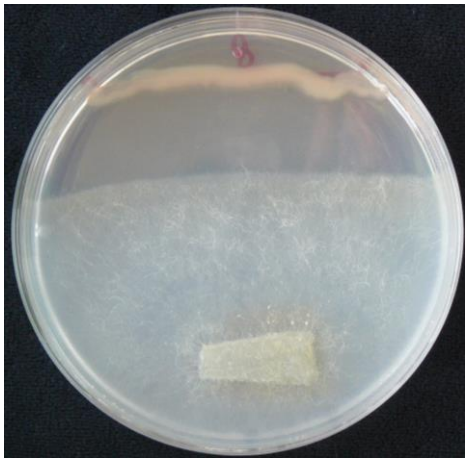

Figure 3. *Lysobacter antibioticus* L08 inhibiting *R. solani* AG2-1 on R2A medium.

Results

Activity against *R. solani* AG2-2IIIB and AG2-1 on R2A

All five *Lysobacter* strains showed *in vitro* activity against *R. solani* AG2-1 and AG2-2 on R2A in a dual culture assay (Fig. 4). When culture filtrates of the *Lysobacter* strains grown in R2B or R2B with 0.2% colloidal chitin were tested for activity on *R. solani* in a similar set-up, or when the culture filtrates were pipetted directly on top of growing mycelium, none of the culture filtrate showed any activity. This indicates that the *Lysobacter* strains are not able to produce chitinases and other inhibitory metabolites under the conditions used for obtaining the culture filtrates, for example the presence of the pathogen is required, or a solid surface or the compounds are rapidly degraded.

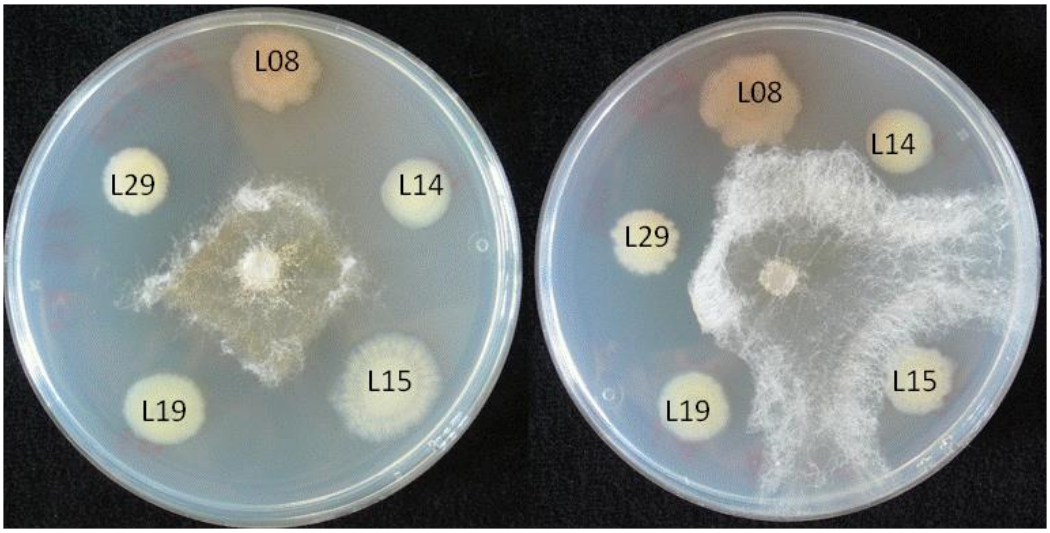

Figure 4. Inhibition by *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 of *R. solani* AG 2-1 (left panel) or *R. solani* AG 2-2 IIIB (right panel) on R2A medium.

In vivo bioassay

To find out if *Lysobacter* strains were also able to suppress *R. solani* disease *in vivo* in cauliflower, strains L08, L14, L15, L19, L29 and a combination of L08, L14 and L15 in a 1:1:1 ratio were added to the conducive Zwaagdijk soil at a density of 10^5 and 10^7 cells/g soil. Germination was not affected by the addition of the *Lysobacter* strains (Appendix 4). After germination, *R. solani* AG2-1 was inoculated and disease progress monitored by determining disease percentage and the distance *R. solani* migrated through the soil. Treatment with most strains did not show a significant different disease percentage, disease distance or area under the disease progress curve (AUDPC) compared to the control (Fig. 5 and Fig. 6). *L. enzymogenes* L29 applied at 10^5 cells/g soil showed significantly decreased disease percentage from the control at 15 days post inoculation (dpi), but this was not seen in the AUDPC. *L. gummosus* L15 applied at 10^7 cells/g soil showed a significantly decreased AUDPC, but this was not seen in the disease percentage or disease distance (Fig. 5 and Fig. 6). However, *L. enzymogenes* L19 applied at a density of 10^5 cells/g soil showed a significant disease suppression for both 15 days post inoculum of *R. solani* disease, distance and AUDPC calculations (Fig. 5 and Fig. 6).

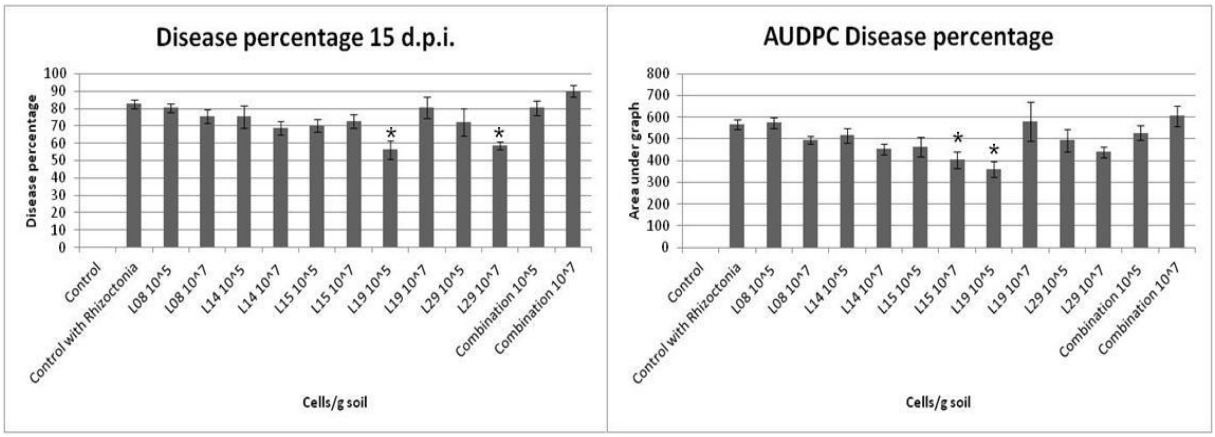

Figure 5. Left panel: disease percentage 15 days post inoculation (dpi) of *R. solani* of cauliflower seeds grown in conducive Zwaagdijk soil supplemented with *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 on *R. solani* at 10^5 cells/g soil (10^5) or 10^7 cells/g soil (10^7). Right panel: AUDPC of disease percentage. An asterisk indicates a significant difference ($p<0.05$) from the control treatment tested with analysis of variance and Dunnet post hoc analysis.

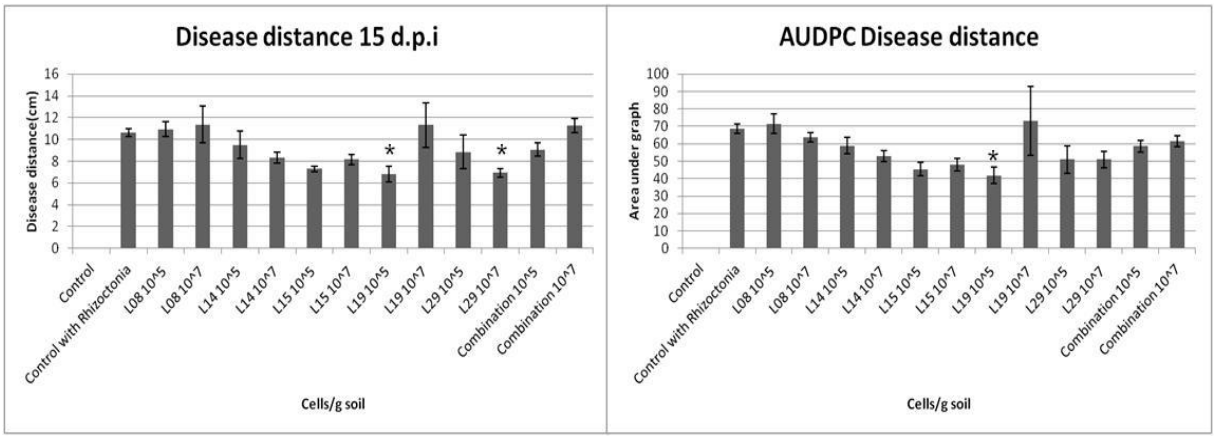

Figure 6. Left panel: disease distance 15 days post inoculation (dpi) of *R. solani* of cauliflower seeds grown in conducive Zwaagdijk soil supplemented with *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 on *R. solani* at 10^5 cells/g soil (10^5) or 10^7 cells/g soil (10^7). Right panel: AUDPC of disease distance. An asterisk indicates a significant difference ($p<0.05$) from the control treatment tested with analysis of variance and Dunnet post hoc analysis.

This bioassay was repeated, with non-rifampicin resistant *Lysobacter* strains. In this experiment strain only strain L14 applied at 10^5 cells/g soil showed a significantly different disease percentage from the control at 15 dpi. However, this disease percentage was higher than the control treatment (Appendix 5 and Appendix 6). These results indicate that none of the strains could suppress the disease development of *R. solani* consistently.

Colonization

To find out if *Lysobacter* treatments can colonize cauliflower *in vivo*, rhizospheres were collected of plants grown for 15 days in Zwaagdijk soil to which rifampicin resistant *Lysobacter* strains L08, L14, L15, L19, L29 and a combination of L08, L14 and L15 in a 1:1:1 ratio were added. Rhizospheres samples were plated on R2A supplemented with rifampicin and colonization was determined by dilution plating (Fig. 7). No bacteria were retrieved from the control treatments. When applied at 10^5 cells/g soil, strains L14 could not be retrieved, whereas L08 L15, L19 and L29 could be retrieved ranging from $3*10^3$ to $1.3*10^6$ cells/g soil. When the combination of L08, L14 and L15 was applied, $1*10^5$ cells/g rhizosphere were retrieved. When applied at 10^7 cells/g soil, L08, L14, L15, L19 and

L29 could be retrieved ranging from $3*10^3$ to $4*10^6$ cells/g soil (Fig. 7). When the combination of L08, L14 and L15 was applied, $7.8*10^5$ cells/g rhizosphere were retrieved.

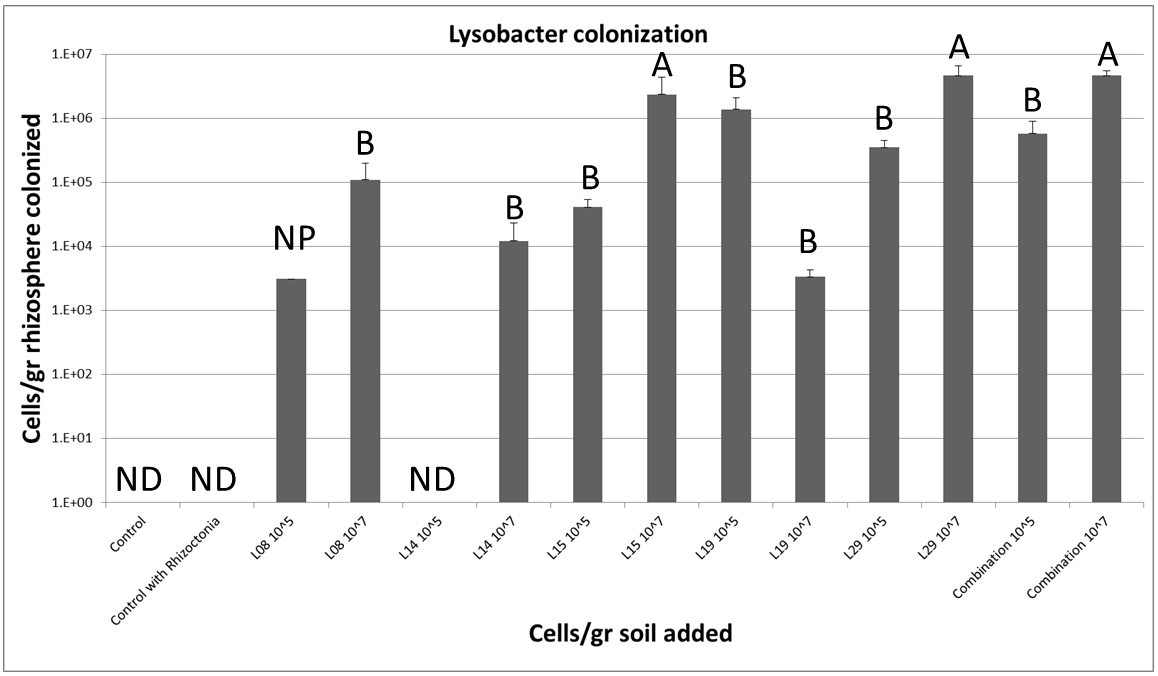

Figure 7. Colonization of *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 collected from the rizosphere of cauliflower. Bars indicated with a different letter indicate a significant difference ($p<0.05$) between the samples tested with analysis of variance and LSD post hoc analysis. ND indicates notdetected. NP indicates that statistics is not possible because there is only one repetition that worked. The detection limit of this experiment is 284 cells/gr rhizosphere.

BOX-PCR confirmed that all bacteria that were isolated were the same strains as applied (Appendix 7), except for strain L14 at both 10^5 and 10^7 cells/gr soil. Overall, this indicates that the all *Lysobacter* strains colonize cauliflower rhizospheres with around 10^5-10^6 cells/g rhizosphere, with the exception of L14, which seemed a poor colonizer. These results indicate that the lack of disease suppression for strain L08, L15 and L29 (Fig 5 and Fig. 6) cannot be explained by a poor colonization. In comparison between 10^5 and 10^7 cells/gr soil treatments showed that inoculation at 10^7 cells/gr soil results in better colonization for *Lysobacter gummosus* L15, *Lysobacter enzymogenes* L29 and the combination of *Lysobacter antibioticus* L08, *Lysobacter capsici* L14 and *Lysobacter* L15. When a combination of L08, L14 and L15 in a 1:1:1 ratio with a concentration of 10^7 cells/g soil per strain was added to the soil higher amount of cells were retrieved from the rhizosphere compared to L08 and L14 applied alone. Potentially the majority of the cells retrieved from the mixture belonged to strain L15, since all the tested colonies follow the same pattern as the L15 control.

Chitinase activity

All strains showed degradation of chitin in the medium (Fig 8). However not all photos showed a clear halo of chitin degradation by the *Lysobacter* species. This indicates that the chitinase(s) are functional in all of the *Lysobacter* strains used.

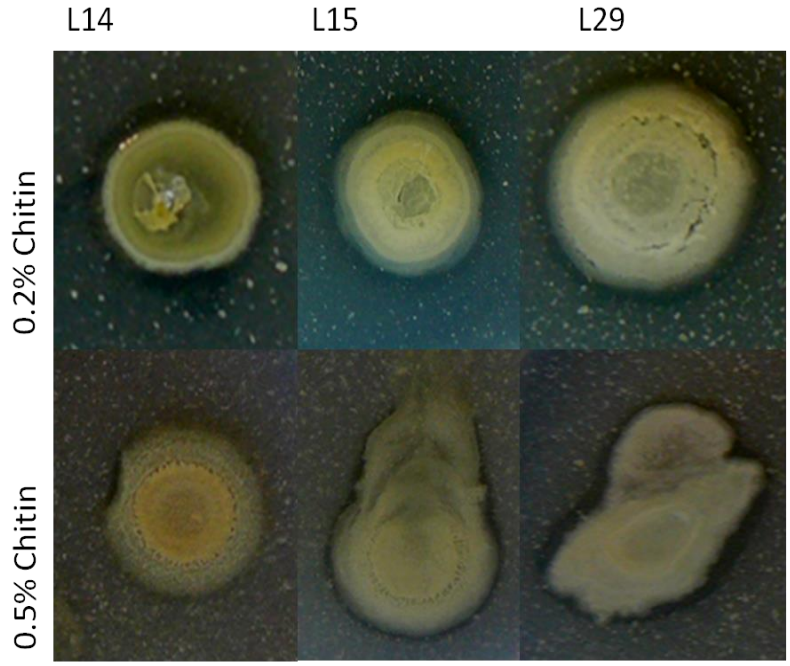

Figure 8. Chitin degredation by *Lysobacter* strains L14: *L. capsici* L14; L15: *L. gummosus* L15 and L29: *L. enzymogenes* L29 on R2A medium with 0.2% and 0.5% added. Photos of *Lysobacter antibiotcus* L08 and *Lysobacter enzymogenes* L19 were unclear and not shown.

Site-directed mutagenesis of the chitinase gene

Site-directed mutagenesis was attempted to test the inhibitory role of chitinases *in vitro*. Flanking sequences are required to be able to delete a complete gene. Even though genome sequences were obtained in November 2012, the flanking sequences for the *chiA* gene of strain L08 and L15 were unavailable. By genome walking it was attempted to retrieve the missing upstream region of the *chiA* gene of strain L08 and L15, however, the genome walking was unsuccessful. Therefore, site-directed mutagenesis has only been performed for L14. Upstream and downstream flanking fragments of the *chiA* gene have joined by overlap extension PCR and ligated into the pEX18Tc vector. Sequencing results from the fragment inserted in pEX18Tc showed an insertion with 2 nucleotide substitutions ,with coding for a different amino acid, into the vector (Appendix 8 and Appendix 9). Introduction of the pEX18Tc-*chiA* to *Lysobacter capsici* L14 was unsuccessful.

Chitinase expression in presence and absence of *R. solani*

To find out if the presence of *R. solani* dead cell material regulates the expression of *chiA* in *Lysobacter* strains L08, L14, L15, L19 and L29. First, the *Lysobacter* strains were grown in R2B to find the best timing for expression analysis by performing RT-PCR. After around 42 h the stationary growth phase was reached (Fig. 9). The *chiA* expression is highest during the exponential growth phase, between 24 and 48 hours after inoculation (Appendix 9).

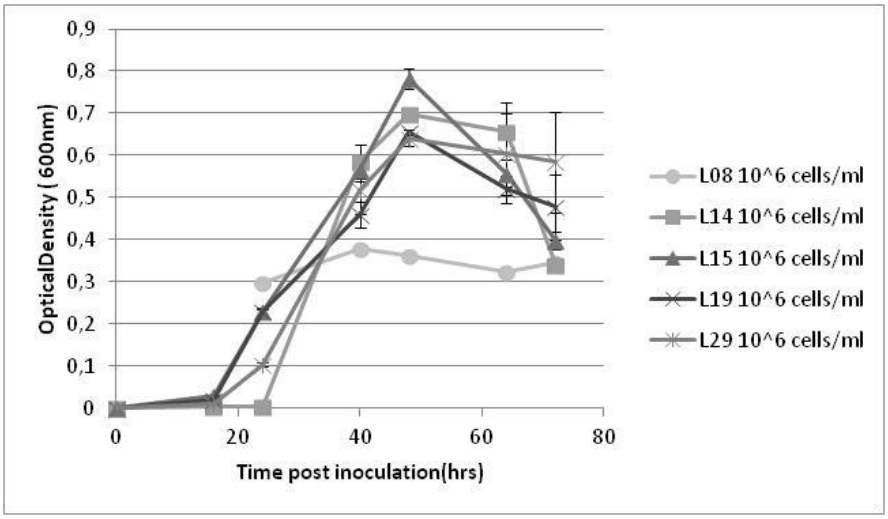

Figure 9. Growth curve of *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 grown in R2B.

Therefore, 24 hours of incubation was selected as the timepoint to analyse the effect of dead *R. solani* AG2-1 tissue by qPCR. When the strains were grown in R2B medium supplemented with dead *R. solani* AG2-1 tissue none of the strains showed a significantly different expression of *chiA*, except for strain L19, (Fig. 10). The *chiA* expression of L19 was significantly lowered in comparison to *chiA* expression when grown on R2B without *Rhizoctonia solani* cell material. This indicates that the cell material of *Rhizoctonia solani* AG2-1 does not significantly influence the expression of *chiA* of most *Lysobacter* strains.

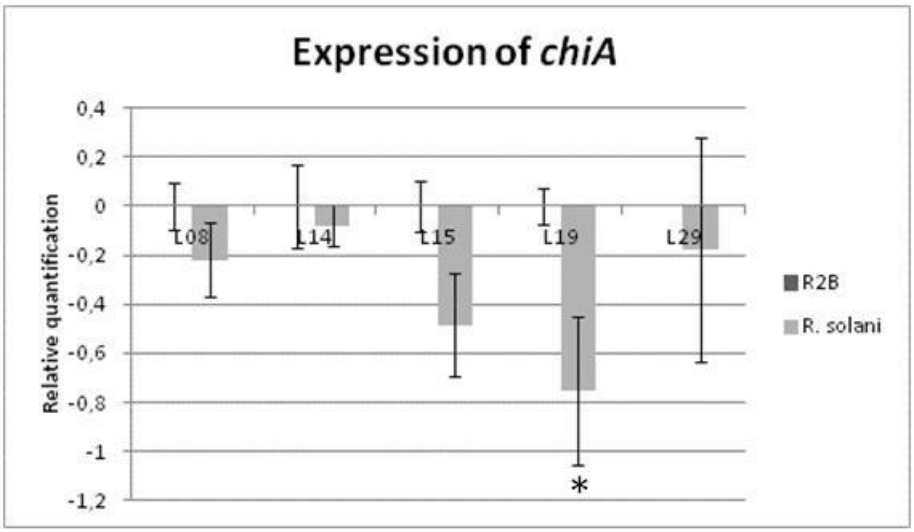

Figure 10. Relative quantification of *chiA* by *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 after 24 hours of inoculation. *Lysobacter* strains grown in R2B supplemented An asterix indicates a sifnificatly different expression when *R. solani* is added. Tested with independent sample T-test

Activity against *R. solani* AG2-2IIIB and AG2-1 on R2A with and without colloidal chitin

To determine the effect of colloidal chitin on antifungal activity of the *Lysobacter* strains, the strains were line-inoculated on medium supplemented with colloidal chitin and *R. solani* AG 2-1 was inoculated. In this set-up the addition of colloidal chitin inhibited the activity of each strain. For L08 and L19 this is only the case when 0.5% colloidal chitin was added (Fig. 11).

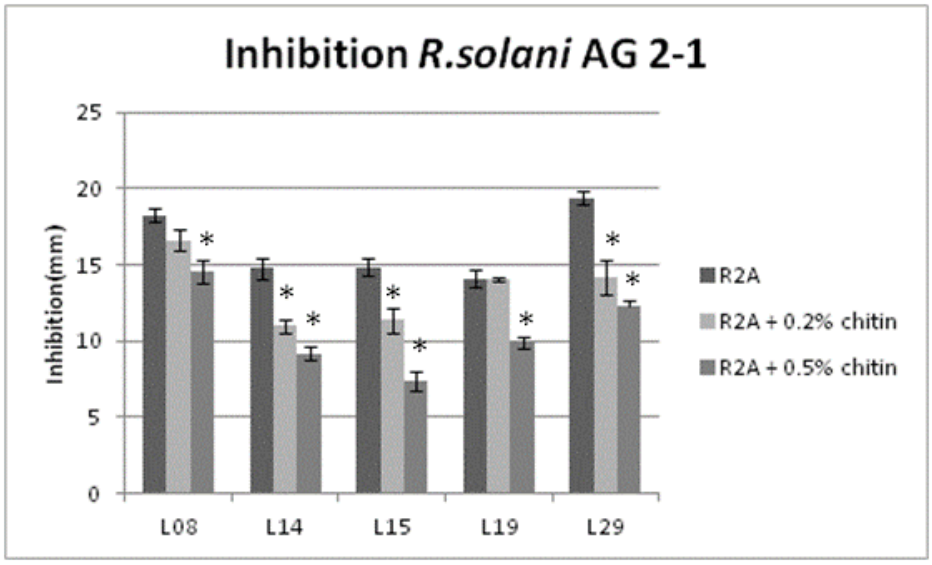

Figure 11. Inhibition in mm of *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29. An asterisk indicates that the treatment differs significantly (P<0.05) from the control (R2A) tested with analysis of variance and Bonferroni post hoc analysis.

In a different set up, where the *Lysobacter* strains were spot inoculated on a plate instead of inoculated in a line, all strains were tested for inhibition against *R. solani* AG 2-1 and *R. solani* AG 2-2 IIIB on R2A, R2A with 0.2% chitin and R2A with 0.5% chitin. *Lysobacter s*trains L14 and L19 and L29 showed a similar decreased inhibition when colloidal chitin was added to the medium as the first set up. *Lysobacter* strains L08 and L15 do not show a decreased inhibition against *R. solani* AG2-1 when colloidal chitin was added. Inhibition of *R.* solani AG2-2 IIIB has only been tested in the second set-up. *Lysobacter* strains L08 and L15 did not show any decreased inhibition against *R. solani* AG2-2 IIIB when colloidal chitin was added. *Lysobacter* strains L14 shows decreased inhibition against *R. solani* AG2-2 IIIB when both 0.2% and 0.5% chitin are added to the medium. *Lysobacter* strains L19 and L29 only show decreased inhibition against *R. solani* AG2-2 IIIB when 0.2% colloidal chitin is added, when 0.5% colloidal chitin is added there is no decreased inhibition(Appendix 10). These results showed that when colloidal chitin is added the antifungal activity of the *Lysobacter* strains was inhibited.

Extraction and HPLC analysis of inhibition zones on R2A with and without colloidal chitin

The inhibition of the activity against *R. solani* AG2-1 by all *Lysobacter* strains in presence of colloidal chitin (Fig. 11) indicates that bioactive metabolites were downregulated. Because the site-directed mutation of the *chiA* gene did not succeed, the role of chitinases could not be determined. Therfore, a HPLC analysis was performed in order to explain the differences in inhibition. The inhibition zones of *Lysobacter* strains growing on R2A with and without colloidal chitin were extracted with ethyl acetate and analyzed by HPLC (Fig. 12). Different metabolites were produced under these different conditions and all strains showed a different HPLC profile. There is a peak around 28 minutes retention time that seems to be common in all treatments except for the treatment with L29. For *Lysobacter antibioticus* L08 two peaks appeared upon 0.2% chitin addition and one peak increased when 0.5% chitin was added. Addition of 0.2% chitin *Lysobacter capsici* resulted in the loss of four peaks. For *Lysobacter gummosus* it is not very clear, 2 different peaks appeared when 0.5% chitin was added, but upon addition of 0.2% these two peaks were not present. For both *Lysobacter enzymogenes* strains only a few different bioactive metabolites could be detected. *Lysobacter enzymogenes* L19 has one peak that disappeared upon addition of chitin. Two other peaks appeared when 0.5% is added. *Lysobacter enzymogenes* L29 shows a decreased production of bioactive metabolite production when chitin is added. When 0.2% chitin is added two peaks disappeared in comparison to R2A, two more additional peaks disappeared when 0.5% chitin was added. The *Lysobacter enzymoges* strains have a lot of bioactive compounds produced in common, although the production is affected differently by chitin. For example the two peaks occurring at 40 minutes retention time are produced for *Lysobacter*

enzymogenes L19 when 0.5% chitin is added whereas these peaks occur on R2A medium and when 0.2% chitin is added for *Lysobacter enzymogenes* L29

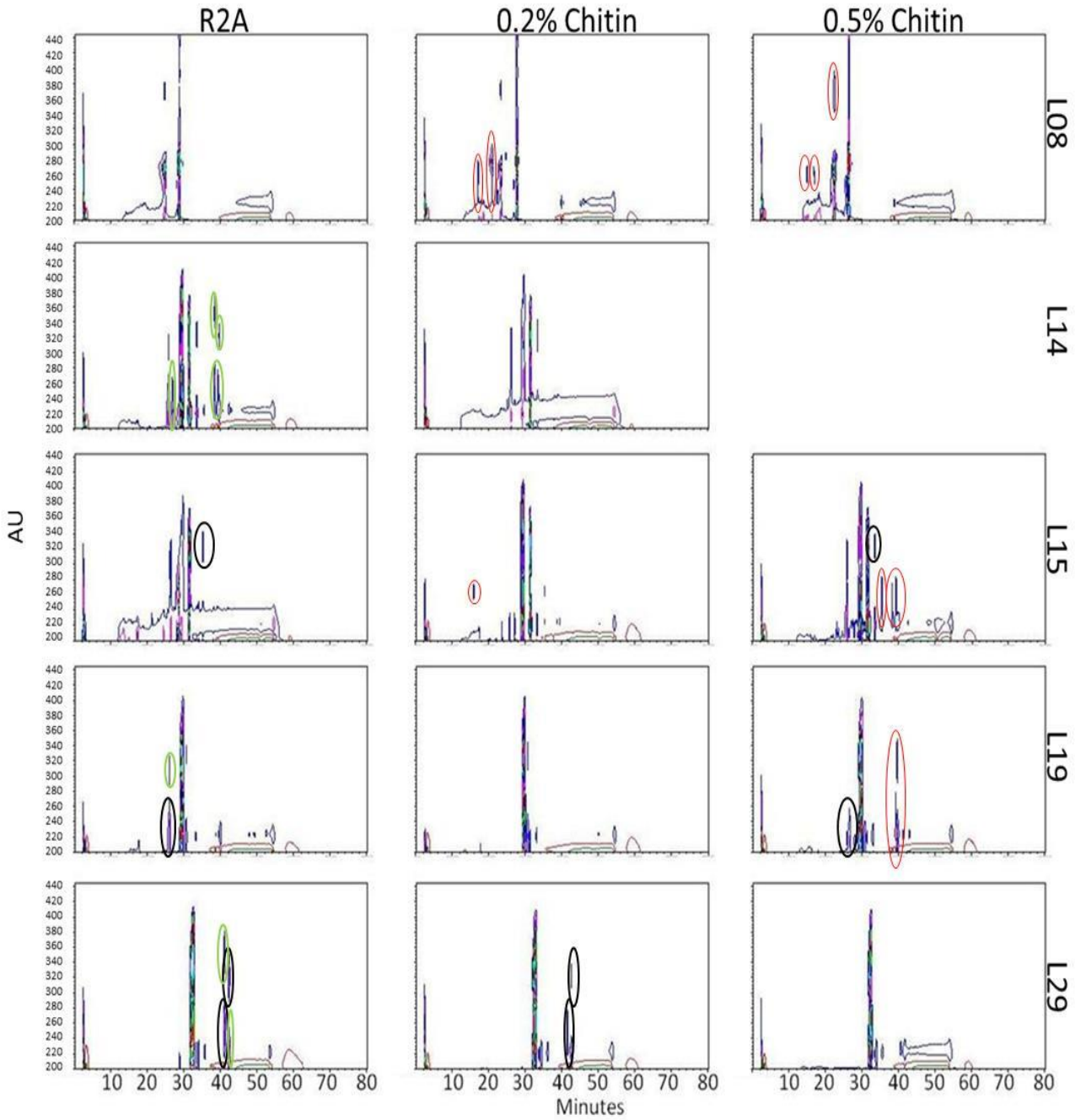

Figure 12 Contour plots of HPLC extracts of inhibition zones of *Lysobacter* strains against *R. solani* AG2-1. *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 grown on R2A, R2A with 0.2% chitin and R2A with 0.5% chitin. The y-axe and x-axe of each graph are equal. Green circles indicate peaks that were only present on R2A medium. Red circles are peaks that were not present in R2A. Black circles are peaks that are present in R2A and only present in only in one of the other medium with chitin, so either present in R2A and R2A with 0.2% chitin or present in R2A and R2A with 0.5% chitin.

This indicates that especially for strains *L. capsici* L14, *L. enzymogenes* L19 and L29 metabolites could be detected that were inhibited by the presence of chitin. For *L. enzymogenes* L29 this relationship was also dose dependent since more peaks disappeared when higher concnetrations of chitin were added.

Discussion

The *Lysobacter* strains inhibit *R. solani in vitro* but do not have *in vivo* activity against *R. solani* in cauliflower. Colonization is necessary for biocontrol activity (Lugtenberg *et al.*, 2001). In previous studies on *Pseudomonas fluorescens* at least 10^5 CFU/g root was required for disease suppression of *Fusarium* wilt (Raaijmakers *et al.*, 1995). Since all *Lysobacter* strains have colonized in at least one treatment at higher concentrations, except for *Lysobacter capsici* L14, it can be expected that the absence of disease suppressiveness is not due to a bad colonization of the *Lysobacter* strains used. This can indicate that either the conditions *in vivo* are not suitable to produce (enough) of their inhibitory metabolites or that when they come in contact with *R. solani* their inhibitory activity is negatively regulated.

Unfortunately no *chiA* mutants were obtained making it more difficult to analyze the role of chitinases in *in vivo* and *in vitro* the disease suppression. A possible explanation for the unsuccessful transformation is the mismatch of two nucleotides, one coding for a different amino acid, making it more difficult for recombination to occur. During transformation, false positive colonies were observed that were resistant to tetracycline while the vector used for site-directed mutagenesis was not present. This indicates that transformation with the pEX18Tc vector is not the most optimal for site-directed mutagenesis. Chitinase purified from *Chromobacterium* could inhibit spore germination of several fungi and a chitinase mutant had a reduced in planta activity against rice blast, tomato leaf blight and wheat leaf rust (Kim *et al.*, 2013). However, a *chiA* mutant of *Lysobacter enzymogenes* strain OH11 lost chitinase activity, but still had activity against *P. capsici*, *P. ultimum*, *S. sclerotiorum*, *R. solani* and *S. cerevisiae* (Qian *et al.*, 2012). This indicates that the inhibition against fungal and oomycete pathogens is not strictly regulated by chitinases and most likely other compounds are involved in antimicrobial activity.

Therefore, the effect of chitin on antimicrobial compounds was further investigated. A qPCR analysis of *chiA* in the presence and absence of dead *R. solani* hyphae did not result in a significant change in the amount of *chiA* transcript levels. This is different from expected since the cell wall of *R. solani* is composed of chitin and the chitinase genes have been known to be upregulated in the presence of chitin (Bhattacharya *et al.*, 2007, Folders *et al.*, 2001). A possible explanation is that *R. solani* cell walls contain compounds that can inhibit the induction of the chitinase genes, a different explanation is that the chitin from the *Rhizoctonia* cell wall is 'masked' or not detected by the *Lysobacter* species. To test whether these compounds are not detected one can test whether addition of colloidal chitin to the medium with *Rhizoctonia* cell wall does increase the expression of *chiA* in *Lysobacter* species.

When chitin was added to the medium the activity of the *Lysobacter* species against *R. solani* was decreased. HPLC analysis showed that the metabolite profile changed when chitin was added to the medium and for *L. capsici* L14 and *L. enzymogenes* L19 and L29 clearly some peaks disappeared upon addition of chitin. This indicates that the presence of chitin can have a negative on the production of antimicrobial compounds. For *Chromobacterium* a similar result was described. When they added chitin to the medium, the chitinase production increased, but the activity against *R. solani* and *Botrytis cinerea* was inhibited (Kim *et al.*, 2013). Also culture filtrates of the *Chromobacterium* grown in presence of chitin showed that *in planta* inhibition of leaf blast, tomato leaf blight and wheat leaf rust was reduced (Kim *et al.*, 2013). Besides chitinases, *Chromobacterium* produced the antimicrobial cyclic lipopeptide chromobactomycin, which was produced less in presence of chitin (Kim *et al.*, 2013). This indicates that there is a co-regulation of chitinases and other antimicrobial compounds. It was speculated that either the processed chitinase substrate, β-1,4-linked N-acetylglucosamine, inhibits the other antibiotics or that other co-regulation between the other antagonistic compounds and *chiA* occurs, including quorum sensing.

Quorum sensing in xanthomonads depend on the Rpf/DSF (Regulation of Pathogenicty factor/Diffusible Signal Factor) and DF (Diffusible Factor) signaling. The extracellular sensor receptor proteins RpfC-RpfG perceive intracellular signals (DSF) and influence gene expression via second messengers, one of them being Clp (cAMP receptor protein (Crp)-like regulator). The globular regulator *Clp* (regulator) regulates the *chiA* expression (Sullivan *et al.*, 2003, Choi *et al.*, 2012) and

has been described to be required for *in vitro* biocontrol activity and antifungal activity of *L. enzymogenes(Kobayashi et al., 2005)*. Furthermore, the Rpf/DSF and DF signalling was proven to be important in the biosynthesis of HSAF (Heat Stable Antifungal Factor) in *L. enzymogenes* (Qian *et al.*, 2013). However,. chitinases and lytic enzymes were not influenced by these signaling pathways (Qian *et al.*, 2013). The role of chitinases in the biocontrol activity of the *Lysobacter* strains used in this study could have been studied more in depth if the site-directed mutagenesis was succesfull. If there is a co-regulation between the *chiA* gene and other metabolites can be investigated by gene expression analyses of genes encoding for the other metabolites in presence and absence of chitin and in the *chiA* mutant.

Future prospects

The HPLC analyses showed that several compounds were downregulated in the presence of chitin in the medium. The compounds with a specific retention time can be extracted from the HPLC analysis and chemical analysis on these compounds can be performed. These compounds can subsequently be tested for their inhibitory function on *R. solani*. Scanning through the genome for possible genes encoding for antimicrobial compounds can be performed as well. If a possible antibiotic gene is identified in the genome a qPCR can be performed in the presence and absence of chitin in order to see if this antibiotic is down or up regulated under these conditions.

In this study we observed that addition of *R. solani* cell wall material does not affect or reduces *chiA* expression. The major cell wall components of *R. solani* are chitin and β1,3-glucan(Tweddell *et al.*, 1994). Most of the *Lysobacters* strains do not increase the *chiA* expression in the presence of *R. solani* , this could be due to the fact that either *chiA* expression is not increased in the presence of chitin or that the chitin from the *R. solani* cell wall is not available to recognize. If *R. solani* masks the chitin fragments it could prevent an increased expression of *chiA*. A qPCR on *chiA* expression in the presence of only colloidal chitin and colloidal chitin plus *R. solani* can reveal whether *R. solani* masks the chitin fragments or whether *chiA* expression is not regulated by chitin.

If *chiA* is causing the inhibition of *R. solani* by the *Lysobacter* species *in vitro* then the focus on the expression should be focused on other lytic enzymes such as β1,3-glucanases or other antibiotics produced.

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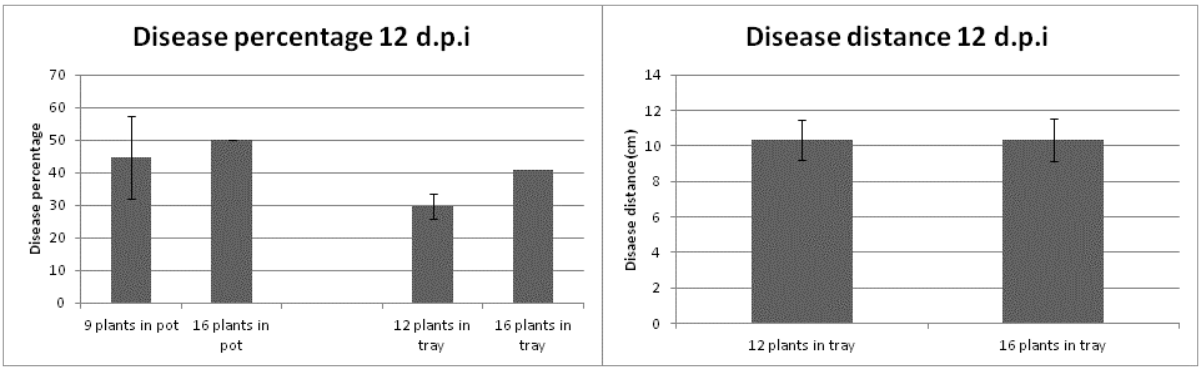
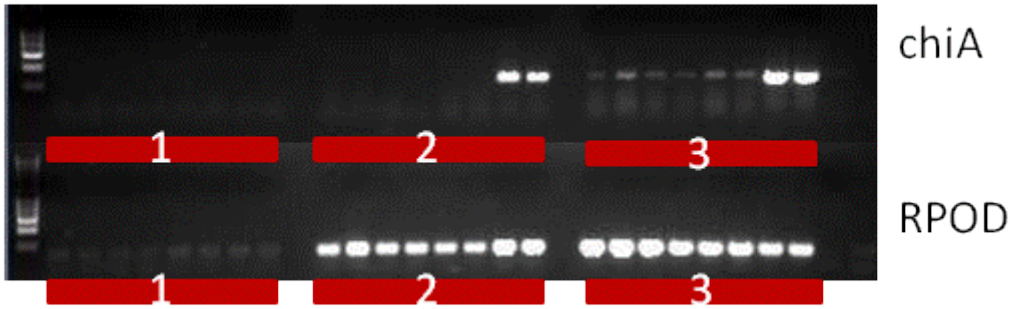
Appendix

Appendix 1 Trial bioassay



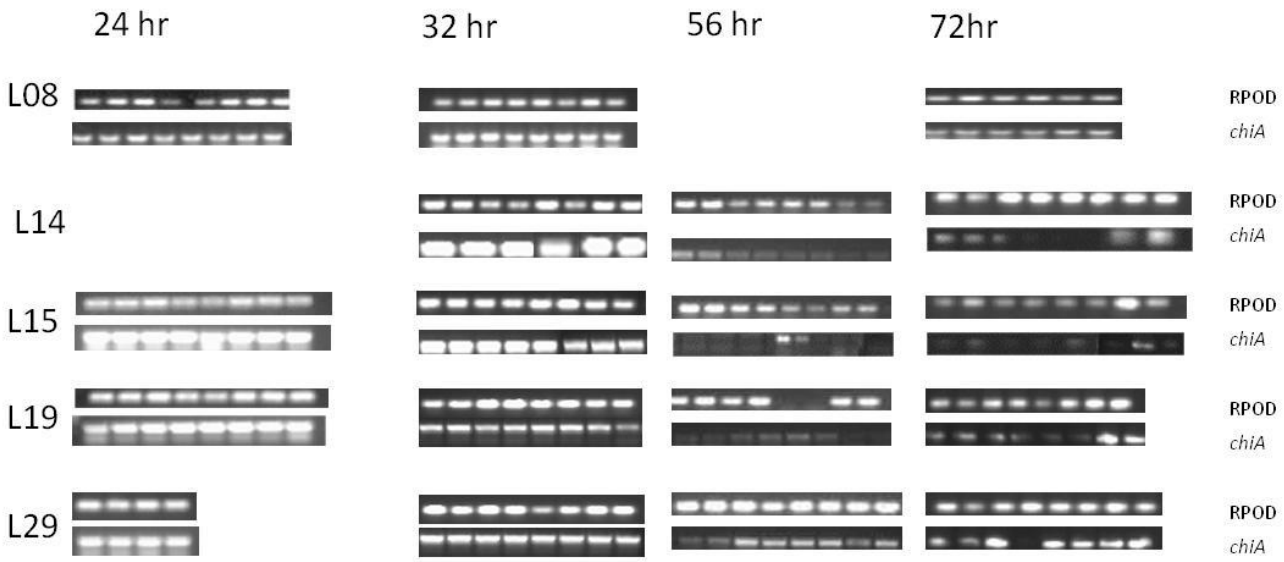
Appendix 1 Trial bioassay pots vs. trays with either 9 or 16 seeds or 12 and 16 seeds sown, respectively. Left panel: disease percentage 12 days post inoculation of *R. solani.* Right panel: disease distance 12 days post inoculation of *R. solani*

Appendix 2 trial number of cycles RT-PCR *chiA*



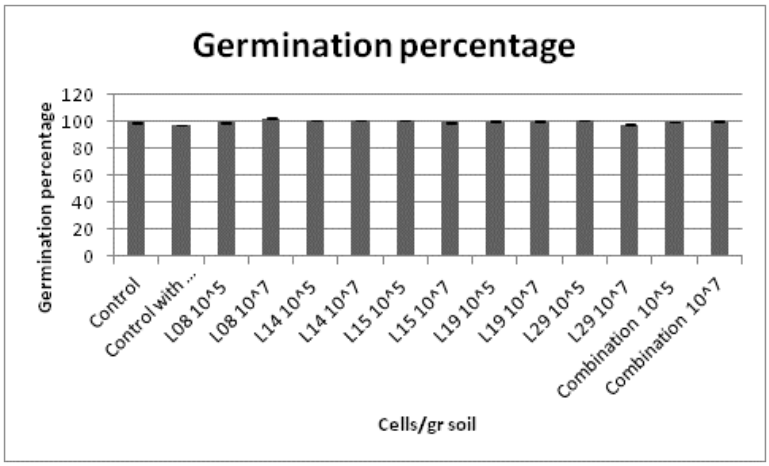
Appendix 2 RT-PCR of *Lysobacter antibioticus* L08 grown on R2B for 72 hours. In order to determine how many cycles are necessary for an RT-PCR, 1 is 25 cycles, 2 30 cycles and 3 35 cycles. Shown are four different PCR reactions loaded in duplicate.

Appendix 3 RT-PCR of *chiA* during growth curve



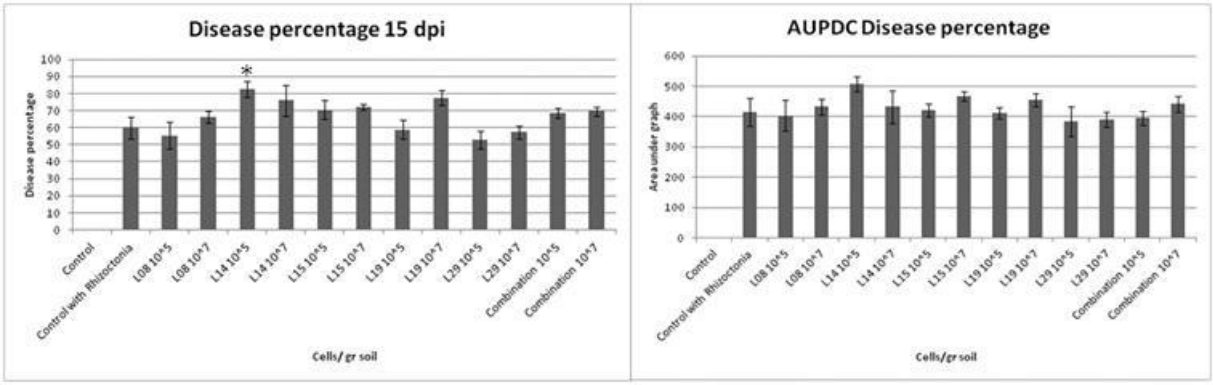
Appendix 3 RT-PCR of *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 grown on R2B, tested genes are *chiA* and the housekeeping gene RPOD at four different time points after inoculation. Missing pictures indicate a RNA isolation that did not succeed. Shown are four different PCR reactions loaded in duplicate.

Appendix 4 Germination percentage bioassay



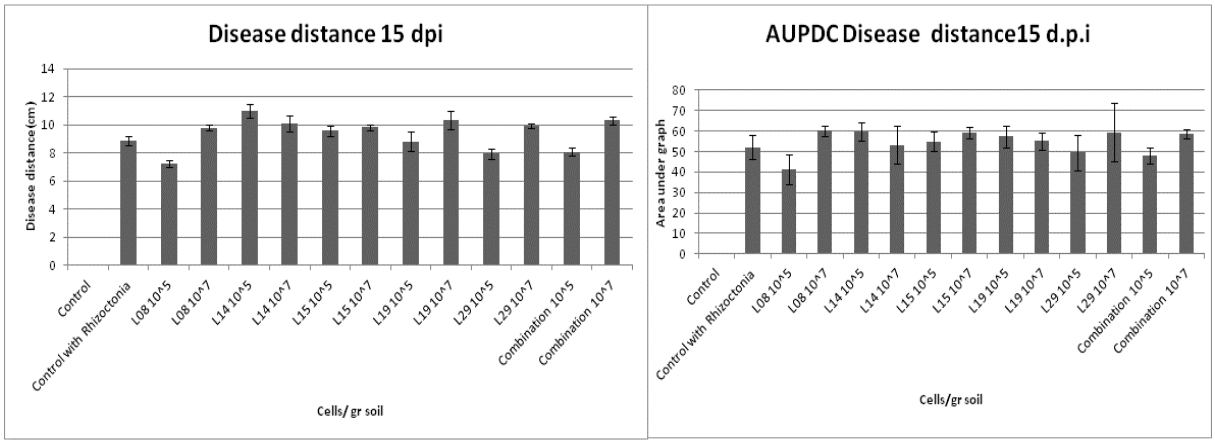
Appendix 4 Germination percentage of cauliflower seeds inoculated with *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 11 days post sowing of the seeds. Germination was not influenced by the treatments.

Appendix 5 Replicate bioassay disease percentage



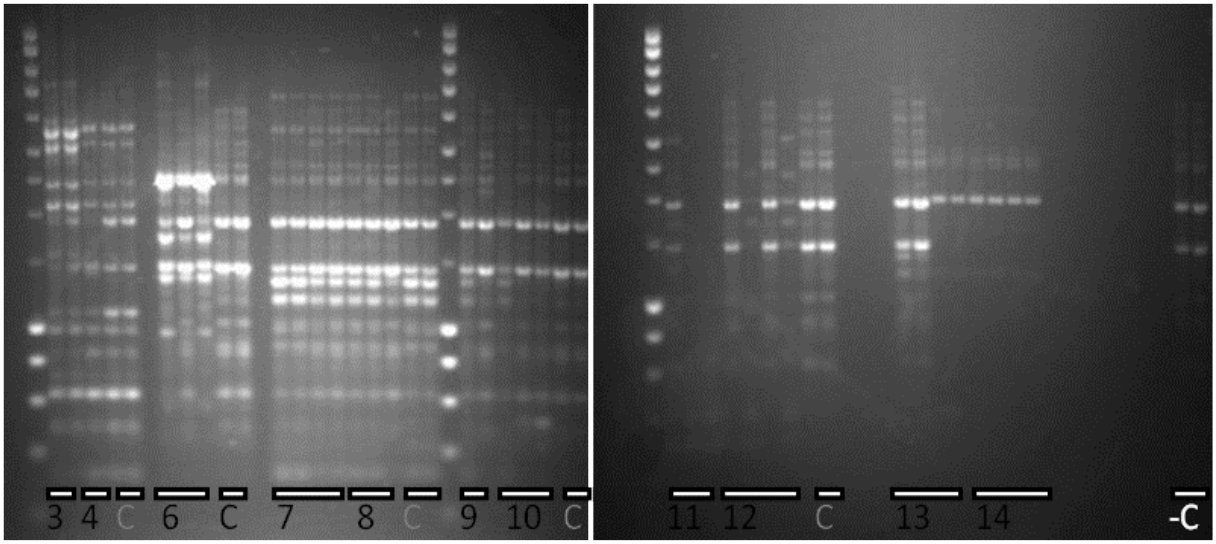
Appendix 5 Left panel: disease percentage 15 days post inoculation (dpi) of *R. solani* of cauliflower seeds grown in conducive Zwaagdijk soil supplemented with different *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 at 10^5 cells/g soil (10^5) or 10^7 cells/g soil (10^7). Right panel: AUDPC of disease percentage. An asterisk indicates a significant difference ($p<0.05$) from the control treatment tested with analysis of variance and Dunnet post hoc analysis.

Appendix 6 Replicate bioassay disease distance



Appendix 6 Left panel: disease distance 15 days post inoculation (dpi) of *R. solani* of cauliflower seeds grown in conducive Zwaagdijk soil supplemented with different *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 at 10^5 cells/g soil (10^5) or 10^7 cells/g soil (10^7). Right panel: AUDPC of disease distancee. An asterisk indicates a significant difference ($p<0.05$) from the control treatment tested with analysis of variance and Dunnet post hoc analysis.

Appendix 7 BOX-PCR bioassay



Appendix 7 BOX-PCR of colonies isolated from the cauliflower rhizosphere of the bioassay. The numbers indicate the treatments from the bioassay section. 3=L08 10^5 cells/gr. soil; 4= L08 10^7 cells/gr; 6= L14 10^7 cells/gr; 7= L15 10^5 cells/gr; 8= L15 10^7 cells/gr; 9= L19 10^5 cells/gr; 10= L19 10^7 cells/gr; 11= L29 10^5 cells/gr; 12= L29 10^7 cells/gr; 13=Combination of L08, L14 and L15 10^5 cells/gr; 14= Combination of L08, L14 and L15 10^7 cells/gr; C=Control of the corresponding treatment(C behind 4 is therefore L08) ; -C=Negative control (miliQ instead of cells). L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29.

Appendix 8 *chiA* fragment sequenced aligned with *chiA* fragment designed, nucleotides

```
chiA designed       CGTGCTCACTTATGTCGAGGACGACGGCGAGATAGAGGCCAGCGAACTCGCGATCGGCCT
chiA sequenced      ------------------------------------------------------------

chiA designed       GACCGGCCGCGGCCAGCGCGACCTCGATGGCCGCGAACTGAGCGTCGTGCATGTCGAGGA
chiA sequenced      ----------------------------------------CGTCGTGCATGTCGAGGA
                                                             ******************

chiA designed       CAAACGCGCATAGCGCCTGCGGCGCGATCAAACGGCGCGCGGCGATCGCTGGAGACTGAT
chiA sequenced      CAAACGCGCATAGCGCCTGCGGCGCGATCAAACGGCGCGCGGCGATCGCTGGAGACTGAT
                    ************************************************************

chiA designed       CGCCGATCCGCGCCCTCCCGCCACGTCTTGGCGTCGCAATGACGCAACCGGACCTGCCCC
chiA sequenced      CGCCCATCCGCGCCCTCCCGCCACGTCTTGGCGTCGCAATGACGCAACCGGACCTGCCCC
                    **** ********************************************************

chiA designed       GACGCTAAGCCGCCATCGCGAGACCTGCGCGCGCCCCCGCTCTCCTTATCGCGCCAGGCC
chiA sequenced      GACGCTAAGCCGCCATCGCGAGACCTGCGCGCGCCCCCGCTCTCCTTATCGCGCCAGGCC
                    ************************************************************

chiA designed       TGCGCCCCAGCCCAGCACGCCTGTGCGCACATTTGTCCTTCGGACATCCGTTTGTCCGTT
chiA sequenced      TGCGCCCCAGCCCAGCACGCCTGTGCGCACATTTGTCCTTCGGACATCCGTTTGTCCGTT
                    ************************************************************

chiA designed       TTGAGGCAAATCGCCGCGCTGCGGCACGTTGCGGCGCAGCAAAACAGGGCATCCACTACC
chiA sequenced      TTGAGGCAAATCGCCGCGCTGCGGCACGTTGCGGCGCAGCAAAACAGGGCATCCACTACC
                    ************************************************************

chiA designed       CACGTTGTCAGGGGCTTCCAGGGCTTTCTGTGAACTGCGGCGCAAAGAATGACGACCTAA
chiA sequenced      CACGTTGTCAGGGGCTTCCAGGGCTTTCTGTGAACTGCGGCGCAAAGAATGACGACCTAA
                    ************************************************************

chiA designed       GTTGACAACGTTGTCAGCCAACTCCAGCTTGCTTCGCATCGCACTCGGTCGAGCACCGTG
chiA sequenced      GTTGACAACGTTGTCAGCCAACTCCAGCTTGCTTCGCATCGCACTCGGTCGAGCACCGTG
                    ************************************************************

chiA designed       AACGAGGCCGCGAGGGAGGCGGAGCGCGGCGGGATCGCGCGGGCTCGCAAGACAGGAACC
chiA sequenced      AACGAGGCCGCGAGGGAGGCAGAGCGCGGCGGGATCGCGCGGGCTCGCAAGACAGGAACC
                    ********************.***************************************

chiA designed       ATCGGCACCAACGCACGGACCGGTTATCGCCCGGTCGCGCCGTTACCGCGGCAGTGCGCG
chiA sequenced      ATCGGCACCAACGCACGGACCGGTTATCGCCCGGTCGCGCCGTTACCGCGGCAGTGCGCG
                    ************************************************************

chiA designed       GCAAGCAATGCATCACCGACGGCGGTTATCCGCCGATCACTCGAGGAGGCCCGAGCCTCC
chiA sequenced      GCAAGCAATGCATCACCGACGGCGGTTATCCGCCGATCACTCGAGGAGGCCCGAGCCTCC
                    ************************************************************

chiA designed       CATGCACGACGGAAGGTTCGAGGGCCTTCCTTCAACACGGGCGGGAACCGCCGCTGGCCG
chiA sequenced      CATGCACGACGGAAGGTTCGAGGGCCTTCCTTCAACACGGGCGGGAACCGCCGCTGGCCG
                    ************************************************************

chiA designed       GCCATGGCCGGCAATGTCTTGACTATGCACGGGAGAGAGGAAGCACCATGAAGTAATCGG
chiA sequenced      GCCATGGCCGGCAATGTCTTGACTATGCACGGGAGAGAGGAAGCACCATGAAGTAATCGG
                    ************************************************************

chiA designed       CCTGACGTGAAAGCCCCTCTCCCGCTCGCGGGAGAGGGGTTGGGGTGAGGGCCGCAAAAA
chiA sequenced      CCTGACGTGAAAGCCCCTCTCCCGCTCGCGGGAGAGGGGTTGGGGTGAGGGCCGCAAAAA
                    ************************************************************

chiA designed       ACCGCAACCGCCGCCCGCACTCACGCGACCCACCCGACGCCATCGCGCGTCGCGAATTTC
chiA sequenced      ACCGCAACCGCCGCCCGCACTCACGCGACCCACCCGACGCCATCGCGCGTCGCGAATTTC
                    ************************************************************

chiA designed       CCGCTTTTGCAAAGCCGTGGCGAGCGATACGCCACGGTGATCTGCAACGGAGCGCCGATC
chiA sequenced      CCGCTTTTGCAAAGCCGTGGCGAGCGATACGCCACGGTGATCTGCAACGGAGCGCCGATC
                    ************************************************************

chiA designed       ACCCCCCCGATCGGCGCTCCGATTTCGTTTTCGGCGGCAGGCACTCGCCTTCGCCCATGG
chiA sequenced      ACCCCCCCGATCGGCGCTCCGATTTCGTTTTCGGCGGCAGGCACTCGCCTTCGCCCATGG
                    ************************************************************
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```
chiA designed       GTCGGCCCGAGCGCGATTCGCGCAACCATGCCTCGCTATCGGCAACGCGATGCCTGTCGG
chiA sequenced      GTCGGCCCGAGCGCGATTCGCGCAACCATGCCTCGCTATCGGCAACGCGATGCCTGTCGG
                    ************************************************************

chiA designed       CTTGGACCATCCGCGCGCGGCGCCACGGCCGTTCGCTCAGCGAGTTTGTCAGCCTGCTCC
chiA sequenced      CTTGGACCATCCGCGCGCGGCGCCACGGCCGTTCGCTCAGCGAGTTTGTCAGCCTGCTCC
                    ************************************************************

chiA designed       GATCGCACGATGCGACGTCCGAGCAAGGCCGAGTCAGCGCACAAGCTCGCGCTTCAGCAC
chiA sequenced      GATCGCACGATGCGACGTCCGAGCAAGGCCGAGTCAGCGCACAAGCTCG-----------
                    *********************************************

chiA designed       ACCACCCGCGTGATCAGCCCGCATACCGATGCGCAGCGGCTCAATCGACCGGCCGCCACA
chiA sequenced      ------------------------------------------------------------

chiA designed       CCGGCTTGCCGTCGCGCCCCGGCACCTGCCGCATCGCCGGCGGCATCACATACAGCGTGT
chiA sequenced      ------------------------------------------------------------

chiA designed       CGAG
chiA sequenced      ----
```

Appendix 8 An overview of the clustering of the *chiA* fragment designed and the and the isolated *chiA* fragment from pEX18Tc sequenced, and asterix indicates that the base pairs between expected and sequenced match. Red nucleotides represent a possible nucleotide substitution during the PCR.

Appendix 9 *chiA* fragment sequenced aligned with *chiA* fragment designed, amino acids

```
chiA designed       RAHLCRGRRRDRGQRTRDRPDRPRPARPRWPRTERRACRGQTRIAPAARSNGARRSLETD
chiA sequenced      ----------------------------------RRACRGQTRIAPAARSNGARRSLETD
                                                      *************************

chiA designed       RRSAPSRHVLASQStopRNRTCPDAKPPSRDLRAPPLSLSRQACAPAQHACAHICPSDIR
chiA sequenced      RPSAPSRHVLASQStopRNRTCPDAKPPSRDLRAPPLSLSRQACAPAQHACAHICPSDIR
                    * ************************************************************

chiA designed       LSVLRQIAALRHVAAQQNRASTTHVVRGFQGFLStopTAAQRMetTTStopVDNVVSQLQ
chiA sequenced      LSVLRQIAALRHVAAQQNRASTTHVVRGFQGFLStopTAAQRMetTTStopVDNVVSQLQ
                    ************************************************************

chiA designed       LASHRTRSSTVNEAAREAERGGIARARKTGTIGTNARTGYRPVAPLPRQCAASNASPTAV
chiA sequenced      LASHRTRSSTVNEAAREAERGGIARARKTGTIGTNARTGYRPVAPLPRQCAASNASPTAV
                    ************************************************************

chiA designed       IRRSLEEARASHARRKVRGPSFNTGGNRRWPAMetAGNVLTMetHGREEAPStopSNRPD
chiA sequenced      IRRSLEEARASHARRKVRGPSFNTGGNRRWPAMetAGNVLTMetHGREEAPStopSNRPD
                    ************************************************************

chiA designed       VKAPLPLAGEGLGStopGPQKTATAARTHATHPTPSRVANFPLLQSRGERYATVICNGAP
chiA sequenced      VKAPLPLAGEGLGStopGPQKTATAARTHATHPTPSRVANFPLLQSRGERYATVICNGAP
                    ************************************************************

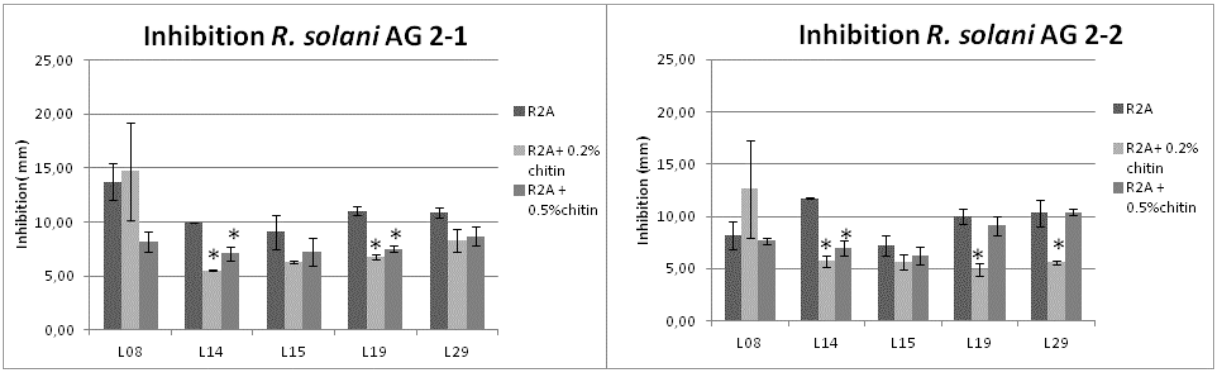
chiA designed       ITPPIGAPISFSAAGTRLRPWVGPSAIRATMetPRYRQRDACRLGPSARGATAVRSASLS
chiA sequenced      ITPPIGAPISFSAAGTRLRPWVGPSAIRATMetPRYRQRDACRLGPSARGATAVRSASLS
                    ************************************************************

chiA designed       ACSDRTMetRRPSKAESAHKLALQHTTRVISPHTDAQRLNRPAATPACRRAPAPAASPAA
chiA sequenced      ACSDRTMetRRPSKAESAHKL---------------------------------------
                    ********************

chiA_designed       SHTACR
chiA_sequenced      ------
```

Appendix 9 An overview of the amino-acid clustering of the *chiA* fragment designed and the and the isolated *chiA* fragment from pEX18Tc sequenced, and asterix indicates that the base pairs between expected and sequenced match. A red amino acid represent a possible mismatch do to nucleotide substitution at the PCR.

Appendix 10 Replicate *in vitro* activity of *Lysobacter* strains against *R. solani* with chitin



Appendix 10 Inhibition(mm) by *Lysobacter* strains L08: *L. antibioticus* L08; L14: *L. capsici* L14; L15: *L. gummosus* L15; L19: *L. enzymogenes* L19; L29: *L. enzymogenes* L29 against *R. solani.* Left panel: AG2-1; Right panel: AG2-2 on R2A, R2A supplemented with 0.2% and 0.5 % colloidal chitin, respectively. An asterisk indicates a significant difference ($p<0.05$) compared to the control (R2A) tested with analysis of variance and Bonferroni post hoc analysis.