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Potential of combined biological control agents to cope with *Phytophthora parasitica*, a major pathogen of *Choisya ternata*

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Abstract *Phytophthora parasitica* infection of *Choisya ternata* can cause important economical loses due to root rot disease. This research focused on testing the potential benefit of chemical treatment (Mefenoxam) and Biological Control Agents (*Glomus intraradices*, *Gliocladium catenulatum, Trichoderma atroviridae* and *Bacillus amyloliquefaciens*) in protecting *C. ternata* against *P. parasitica*. BCAs were applied as individual and/or combined treatments. The effect of the treatment was observed by monitoring *C. ternata* symptoms. A real-time PCR targeting the *ypt1* gene was also adapted to evaluate *P. parasitica* development in the substrate. The use of Mefenoxam provided the higher level of plant protection. However, a significant reduction in plant symptoms and *P. parasitica* development was also observed with the combined treatment of *G. intraradices* with *G. catenulatum* and *G. intraradices* with *T. atroviridae*. Another combined treatment with *G. catenulatum* and *B. amyloliquefaciens* increased the pathogen density and severity. No individual treatment had a significant effect on the pathogen. Our results highlight the potential of biological control in protecting *C. ternata* against *P. parasitica* and the advantage of combined strategies.

Keywords Biocontrol agents · Choisya ternata · Chemical treatment · Phytophthora parasitica · Root rot disease · Soil-borne pathogens

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Introduction

Choisya ternata (Mexican orange) is an average shrub that occurs naturally on the rocky slopes of the USA and Mexico (Boyd et al. 2007; Talbot and Wedgwood 2009) and is also widely used as an ornamental and landscape plant for decorative purposes. The production of this plant in nursery pots makes it more sensitive to root rot caused by soil microorganisms, such as *Phytophthora* spp., *Pythium* spp., and *Fusarium* spp., among others, with *Phytophthora* spp. dominating the pathogen species isolated by the Food and Environment Research Agency (Fera) plant clinic. These soil-borne pathogens lead to a loss of *Choisya* vigor and marketability, resulting in heavy economic losses up to 80% in some cases for horticulturists (Astredhor – French technical institute for horticulture – Cannesan 2017, internal communication; Talbot and Wedgwood 2009). A first reported case of *Choisya* root

rot caused by *Phytophthora* was reported by the Istituto Regionale per la Floricoltura in San Remo in 2000, Italy. In 2005, in the Lorraine region (east of France), *P. parasitica* was isolated from *C. ternata* roots. Several *Phytophthora* species (*Phytophthora citricola*, *Phytophthora cryptogea*, *Phytophthora cinnamomi*, *Phytophthora citrophthora* and *Phytophthora parasitica)* have been then isolated and identified from *Choisya* by the plant clinic of the Royal Horticultural Society (RHS). The presence of more than one pathogen on a single plant has been reported at ADAS (national agricultural advisory service of the Ministry of Agriculture, Fisheries and Food in UK), such as *Phytophthora* spp. and *Pythium* spp. (Talbot and Wedgwood 2009). Finally the Experimental Station for Plants and Flowers (GIE) in south-west France identified *P. parasitica* as the major pathogen in *Choisya* affected by root rot (Talbot and Wedgwood 2009).

Phytophthora is an oomycete that is responsible for many destructive diseases in plants worldwide (Larousse and Galiana 2017). More than 100 species of *Phytophthora* have been identified, and their number is still increasing (Kroon et al. 2012; Meng et al. 2014). This species is responsible for numerous agricultural and ornamental disasters (Haas et al. 2009; Grünwald et al. 2012; Larousse and Galiana 2017). Some species of the Rutaceae family are hosts of *Phytophthora*. For example, *P. parasitica*, a soil-borne pathogen, causes serious root rot damage in Citrus, Mexican orange and orchards in nurseries throughout the world (Vernière et al. 2004; Boava et al. 2011; Benfradj et al. 2016). Most *Phytophthora* diseases are soil-borne and difficult to control. Different strategies are used to control the disease (Drenth et al. 2006), such as cultural practices to provide good drainage (Hansen 2008) and fungicides (Parra and Ristaino 2001). However, oomycetes, including *Phytophthora*, increasingly develop resistance to chemical products, making the products ineffective (Davidse et al. 1981; Parra and Ristaino 2001; Keinath 2007; Larousse and Galiana 2017). Furthermore, the growing awareness of the adverse effects of pesticides on human health and the need to protect the environment have led to an increase in legislative constraints to reduce their use (Gullino and Kuijpers 1994), such as the Ecophyto French national plan. Taken together, these factors increase the need to develop alternative solutions for pathogen management.

Biological control agents (BCAs) are promising and sustainable alternative solutions for the management of soil diseases (Shen et al. 2007; Li et al. 2012; Liu et al. 2013). Different groups of organisms have been found to exhibit antagonistic activity against pathogens. One group that shows particular potential comprises the arbuscular mycorrhizal fungi (AMF). These microorganisms are capable of colonizing more than 80% of plant species and are naturally present in the soil. In addition to their ability to control pathogens, AMF offer other benefits, including enhanced nutrition and drought tolerance (Vigo et al. 2000; Cameron et al. 2013). AMF may have different mechanisms for biocontrol, such as improving the nutrient status of the plant (Karagiannidis et al. 2002; Wehner et al. 2010), competitive interactions with pathogens (Filion et al. 2003; Wehner et al. 2010), anatomical and architectural changes in the root system (Matsubara et al. 1995; Wehner et al. 2010), microbial community changes in the rhizosphere (Larsen et al. 2003; Wehner et al. 2010), activation of plant defense mechanisms (Pozo et al. 2009; Wehner et al. 2010) and induction of systemic resistance (Cameron et al. 2013). The AMF *Glomus intraradices* was found to reduce disease severity in pea caused by *Aphanomyces euteiches* (Bodker et al. 1998) and root mortality in pepper plant caused by *Phytophthora capsici* (Zheng et al. 2005). The crude extract of the growth medium of *G. intraradices* significantly reduces conidial germination of *Fusarium oxysporum* (Filion et al. 1999), and the exudates from tomato roots colonized by *G. intraradices* provoke the repulsion of *P. parasitica* (Lioussanne et al. 2008). Species of *Trichoderma* and *Gliocladium* are filamentous fungi belonging to the subdivision of Deuteromycetes. These microorganisms display resistance to many toxic compounds, including herbicides, fungicides and phenolic compounds, making it possible to use them as a complementary treatment with pesticides (Smith et al. 1990; Benitez et al. 2004; Naher et al. 2014). These species have been shown to provide different levels of biological control against different soil-borne plant pathogens, including oomycetes, such as *Pythium* spp. and *Phytophthora* spp. (Smith et al. 1990; Naher et al. 2014). *Trichoderma* species can act by competing for nutrients and space (Benitez et al. 2004), plant growth enhancement (Harman et al. 2004b), activation of plant defense mechanisms (Harman et al. 2004a, b; Vinale et al. 2008), and mycoparasitism (Steyaert et al. 2003; Vinale et al. 2008). *Trichoderma* and *Gliocladium* spp. are able to reduce the damage caused by *Phytophthora cactorum* in apple plants (Smith et al. 1990). Other organisms, such as plant growth-promoting rhizobacteria (PGPR), also offer promising potential for controlling pathogens due

to their ability to colonize the rhizosphere, promoting plant growth and inducing systemic resistance (ISR) (Lee et al. 2015; Zhang et al. 2016). PGPR can activate different plant defense mechanisms, such as the oxidative burst, reinforcement of the cell wall, accumulation of secondary metabolites and activation of defense-related enzymes (Conrath et al. 2006; Lee et al. 2015). *Bacillus amyloliquefaciens* is a PGPR with an interesting potential as a BCA. Different strains of *B. amyloliquefaciens* have shown antifungal activity against *Rhizoctonia solani*, *Fusarium oxysporum* and *Sclerotinia sclerotiorum* (Souto et al. 2004), effectively induced resistance against *Phytophthora cactorum* in *Panax ginseng* plants (Lee et al. 2015) and inhibited *Phytophthora capsici* growth (Zhang et al. 2016).

The aim of this study was to investigate the efficiency of different approaches including chemical treatment and BCAs for limiting the development of *P. parasitica* and protecting *C. ternata* from roots rot. To our knowledge, no study has explored the use of BCAs to protect *C. ternata* nursery culture from this pathogen. An experiment close to the production conditions with artificial inoculation of the pathogen and chemical or BCAs application on *Choisya* plants was set up to monitor disease spread and evaluate symptoms intensity. A real-time PCR assay, targeting the ras-related protein gene (*ypt1*) was adapted to evaluate the impact of chemical and BCAs application on *Phytophthora* density in the cultivation substrate of *Choisya*.

Materials and methods

Identification of *Phytophthora* spp. pathogens of *C. ternata*

Isolation of Phytophthora from C. ternata roots

C. ternata plants presenting characteristic *Phytophthora* symptoms were collected from the experimental station (Astredhor stations in the French region Ile de France). The isolation protocol was adapted from the protocol described by Drenth and Guest (2004). Briefly, roots were collected from infected plants and washed with tap water to eliminate all substrate. The roots were then incubated for 6 days at 25 °C in Petri dishes containing 10 ml sterile deionized water and three flower petals from white *Dianthus caryophyllus* buds (white carnation buds). Translucent petals infected with *Phytophthora* were transferred and incubated for 6 days at 25 °C on 1.7% (*w*/*v*) cornmeal Agar (CMA) medium supplemented with 100 mg.L^{-1} pimaricin, 50 mg L^{-1} polymyxin B and 50 mg L^{-1} ampicillin. *Phytophthora* mycelia were then transferred to new 1.7% (*w*/*v*) CMA medium. The final step was repeated twice to eliminate any possible contamination.

Phytophthora strain maintenance

Phytophthora spp. cultures were maintained as described by Attard et al. (2014) and Galiana et al. (2005). *Phytophthora* spp. was maintained on clarified V8 juice agar at 25 °C in the dark. V8 juice was first clarified by mixing it with 15% (*w*/*v*) CaCO3 and centrifuged at 2000 g for 10 min. Next, the supernatant was diluted four times, followed by the addition of 1.5% (w/v) agar and autoclaving. *Phytophthora* was transferred onto new medium every 4–6 weeks. For *Phytophthora* DNA extraction, five small agar plugs from clarified V8 subcultures were placed in a Petri dish of liquid clarified V8 juice and incubated at 25 °C under continuous light for 1 week. The developed mycelium was then collected, cut, washed with sterile deionized water and stored at −20 °C until use.

Phytophthora DNA extraction and quantification

Total DNA was extracted from 50 mg of the *Phytophthora* mycelium using the PowerPlant DNA Isolation Kit (MoBio Laboratories, 121 Carlsbad, CA, USA) according to the manufacturer's instruction with one modification during the homogenization step. This step was performed twice using the FastPrep instrument at 6.5 m/s for 30 s. The DNA concentration were assayed by fluorimetry using the Quant-iTTM dsDNA Broad-Range Assay Kit (Picogreen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA solutions were then stored at −20 °C until use.

Molecular identification of *Phytophthora* strains isolated from infected plants

Phytophthora species were identified using the ITS region, which was considered a barcode not only for fungi but also oomycetes (Xu 2016; Bellemain et al. 2010). Extracted DNA from each of the isolated strains was amplified using the universal PCR primers ITS1

and ITS4 (White et al. 1990). PCR was performed in a 50-µl volume containing 2 ng.µl^{-1} of DNA, 1X of master mix GoTaq green (Promega), and 10 µM of primers ITS1 and ITS4 in the Gene amp® PCR system 9700 (Applied Biosystems). The cycling protocol was 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. Successful amplification was confirmed by electrophoresis on a 1% (*w*/*v*) agarose gel stained with 0.1 µg/ml ethidium bromide. The 600-bp PCR product was sequenced by Genoscreen, Lille, France. Finally, the sequences were blasted against NCBI to identify *Phytophthora* species.

Molecular tools for *Phytophthora* spp. quantification

Primer specificity to Phytophthora

Genus-specific primers were chosen from the available published literature to quantify all the detected species of *Phytophthora* in a single PCR run. The PCR primers were YPh1F (5'-CGACCATKGGTGTGGACTTT-3′) and YPh2R (5'-ACGTTCTCMCAGGCGTATCT-3′), which generate an amplicon of 450 bp. These primers target the single-copy gene *ypt1,* which encodes a protein involved in vesicle transport between endoplasmic reticulum compartments (Chen and Roxby 1996), and they show high specificity for the genus *Phytophthora* (Schena et al. 2006).

Real-time PCR assay of *P. parasitica* isolate DNA

Real-time PCR was tested and optimized to obtain a 90% minimum efficiency using a LightCycler 480 real-time PCR system (Roche, Basel, Switzerland). The reaction conditions were optimized, such as the annealing temperature of the primers and the DNA concentration. Different concentrations of the following two PCR additives were tested: bovine serum albumin (BSA) (New England BioLabs, Ipswich, Massachusetts, USA) and the T4 bacteriophage Gene 32 product (T4 gp 32) (MP-Biomedicals, Santa Ana, CA, USA). Real-time PCR amplification was performed in a 25-µl reaction volume containing 4 ng of isolate DNA; 1 and 5 µm of each primer; 0.5, 1, 1.25, and 1.5 mg ml^{-1} BSA or 10, 15, and 20 ng ml^{-1} T4; 1X Absolute PCR SYBR Green Mix from ThermoScientific or 1X LightCycler® 480 DNA SYBR Green I Master mix (Roche, Basel, Switzerland); and 3 mM, 3.5 mM, and 4 mM MgCl2. The PCR cycling conditions were 95 °C for 15 min, followed by 50 cycles at 95 °C for 15 s, 58, 60 or 61 °C for 45 s and 72 °C for 30 s. A final melting curve step was performed by heating the samples to 95 °C, cooling to 61 °C and slowly heating to 97 °C, increasing the temperature by 1.1 °C every 10 s with continuous measurement of fluorescence at 520 nm. The DNA standard curves were obtained from 10-fold dilutions of DNA from *P. parasitica* INRA-310 obtained from the Institute Sophia Agrobiotech, Nice, France. The PCR efficiency and DNA concentrations were determined using the Roche second derivative maximum method. Real-time PCR reactions for the standard curve were repeated at least three times, and PCR assays always included a negative control (no DNA).

Optimization of the real-time PCR assay for the substrate matrix

To quantify *P. parasitica* in the substrate matrix, we used the real-time PCR protocol with the highest efficiency for the *P. parasitica* isolate DNA. Ten-fold serial dilutions of *P. parasitica* isolate DNA was mixed with DNA extracted from substrate matrix without *Phytophthora*. Quantitative PCR amplification with and without the addition of substrate DNA was performed in the same PCR microplate. The *YPT1* copy number was compared between mixed and pure *P. parasitica* isolate DNA. The DNA standard curves were obtained as described above.

Assay of different treatment against *Phytophthora* root rot in *C. ternata*

Plant materials

C. ternata in 0.4-L pots were obtained from the André Briant Jeunes plants nursery. Plants were re-potted in 4-L SUB21 SOPARCO pots with substrate composed of 30% Irish blond peat F1, 40% Irish blond peat F2, 15% hortifiber M, 15% medium coconut fiber, 1.2 kg.m-3 PgMix 14–16-18 and 5 kg.m-3 Hi END fertilizer. The pots were covered with a jute disk and irrigated with water at pH 6 using micro-sprinklers.

Treatment modules

Tested treatments of *C. ternata* against *P. parasitica* included a chemical treatment with commercial product

(SUBDUE GOLD) and biological treatment with four different BCAs. The application of each species or strain is described in Table 1. One negative control and nine different modules were chosen, and their composition is described in Table 2. The chemical treatment module contained mefenoxam, and the biological treatment modules contained 1 or 2 BCAs as described in Table 2.

Preparation of the *P. parasitica* inoculum and artificial inoculation of substrate

The rice grain inoculum was prepared as described by (Holmes and Benson 1994; Loyd et al. 2014). Twenty-five grams of rice grain and 18 ml of deionized water were placed in flasks and autoclaved twice. Next, three 7-mm-diameter disks of freshly developed *P. parasitica* INRA-310 mycelium growing on a 1.7% (*w*/*v*) CMA were placed in each flask. The flasks were incubated at 25 °C in the dark and shaken twice every day for 1 week. The inoculum consisted of 120 1-week old rice grains, which were artificially inoculated in each substrate pot. Three 5-cm-deep holes were created in the substrate for each pot, and the *Phytophthora* inoculum was divided into the holes. Non-colonized sterile rice grains were used for the negative control. The inoculum concentration was chosen based on preliminary experiments (data not shown).

Symptom notation

Symptoms of *C. ternata* were classified into three different categories (Fig. 1): healthy plants were noted as (h), plants with moderate symptoms (fading leaf colors, more or less yellowish) were noted as (m) and plants with severe symptoms (bronze-colored leaves and loss of turgidity, leading generally to plant death) were noted as (s).

Bioassay in the experimental station

Thirty *C. ternata* in 0.4-L pots for each module (300 plants in total) were received from the André Briant Jeunes plants nursery at the experimental station (Saint Germain en Laye, France). The plants were repotted and treated as explained in Table 2. Every module was randomly divided to two groups (I and II) of 15 plants, each separated by a buffer zone. After one month, all the plants in group I were artificially inoculated as described above, and all the plants in group II were maintained as a control. One month after artificial inoculation, the symptoms of all plants were noted according the scale described above (Fig. 1). In parallel, three plants for every module in group I were randomly selected to quantify the development of *P. parasitica* in the substrate using the developed real-time PCR assay.

Substrate sampling

The substrate of each selected plants was sampled, homogenized and sieved to a particle size of 2 mm. Three 0.4 g samples from each pot were then collected in 2-ml Eppendorf tubes and treated with liquid nitrogen prior to storage at −80 °C until use.

Table 1 Biological control agent composition, applications, doses and ways of applications

Composition	Applications			Doses	Application methods
	Re-potting (R)	(R) + 15 days	(R) + 30 days		
Mefenoxam	X		X	20 $L.ha^{-1}$	Irrigation
G. catenulatum J1446, 320 g/kg: 2.10^8 UFC/g	X		X	0.2 $g.L^{-1}$	Irrigation
G. Intraradices strain 1, 4000 spores/g	X			0.04 $g.L^{-1}$	Incorporation
G. Intraradices 2	X			4 $g.L^{-1}$	Incorporation
T. atroviridae	X			3 $g.L^{-1}$	Incorporation
B. amyloliquefaciens IT45, >109 UFC/g	X	X	X	0.15 $g.L^{-1}$	Irrigation

Table 2 Composition of each treatment module

Codes	Descriptions
T1	No treatment
T2	Mefenoxam
A	*G. catenulatum* and *G. intraradices* strain 1
B	*T. atroviridae* and *G. intraradices* strain 1
C	*B. amyloliquefaciens* and *G. intraradices*
D	*G. Intraradices* strain 2
E	*T. atroviridae*
F	*B. amyloliquefaciens*
G	*G. intraradices* strain 1
H	*G. catenulatum* and *B. amyloliquefaciens*

DNA extraction from substrate samples and quantification of the *Phytophthora* density by real-time PCR

Total substrate DNA was extracted from 0.4 g of moist substrate using the Fast DNA spin Kit for Soil (MP-Biomedicals, Santa Ana, CA, USA) according to manufacturer's instructions with some modifications. First, centrifugation was performed for 20 min, and a facultative incubation was performed for the DSE step. After extraction, the DNA concentration was assessed by fluorimetry using the Quant-iTTM dsDNA Broad-Range Assay Kit (Picogreen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The DNA extracts were stored at −20 °C until use. *Ypt1* copy numbers per gram of dry substrate were estimated for each sample using the real-time PCR conditions as determined above. Several DNA optimal concentrations for the real-time PCR assay were tested, and a concentration of 2 ng μL^{-1} was finally chosen. DNA standard curves were obtained from 10-fold serial dilutions of *P. parasitica* INRA-310 DNA and were performed in triplicate for each run.

Data analyses

The second derivative maximum method developed by Rasmussen (2001) was used to calculate Cq values for each PCR reaction. Construction of the calibration curve that related a known quantity of *P. parasitica* DNA to Cq allowed us to determine the *P. parasitica ypt1* copy number. Amplification efficiencies ( ) were calculated from the slope (s) of the standard curve using = (10–1/s-1). The Shapiro-Wilk test was used to test the *P. parasitica ypt1* copy number normality, and Brown-Forsythe's test was used to test the variance homoscedasticity. A comparison of the *P. parasitica ypt1* copies number quantified between all tested treatments was performed using the parametric Dunnett's test. Pearson's rho correlation was used to describe the link between the pathogen and observed symptoms in plants. All tests were performed with GraphPad PRISM 7, and the statistical significance was set at $p < 0.05$.

Results

Identification of strains isolated from infected plants

Based on the NCBI GenBank blast results for the amplified ITS sequences, two different isolated *Phytophthora* species were identified (Sup. datas 1 and 2). The most dominant species retrieved from infected plants originating from the region Ile-de-France was *P. parasitica*. The second identified species was *Phytophthora tropicalis*. Other pathogenic oomycetes were also found, such as

Fig. 1 Symptoms severity observed after 1 month of inoculation with *P. parasitica*. (h) healthy plant with no stress symptoms, (m) plants with moderate symptoms and (s) plants with severe symptoms

Pythium spinosum, *Pythium intermedium* and *Pythium cylindrosporum* (Sup. datas 1 and 2).

Development of the *Phytophthora* real-time PCR assay

YPh1F/YPh2R primer sensitivity and real-time PCR assay using pure P. parasitica DNA

The YPh1F/YPh2R PCR primers are specific for the *Phytophthora* genus according to Schena et al. 2006. The best results were obtained with a reaction mixture and amplification conditions adapted from Schena et al. 2006 and optimized. The optimized reaction mixture contain 1 μm of each primer, 0.5 mg ml^{-1} BSA, 1X Absolute PCR SYBR Green Mix from ThermoScientific, no T4 and no supplementary $MgCl_2$. The chosen conditions for primers amplification were 61 °C for 45 s. The melting curve indicates the presence of two amplicons when using DNA from *P. parasitica*, a principal amplicon with a Tm of 86 °C and a second amplicon at 83 °C. Different modifications of the reaction mixture and amplification conditions did not reduce or prevent the second amplification. This second amplicon did not appear when *P. tropicalis* was amplified under the same conditions. This conditions allowed us to achieve an efficiency that consistently exceeded 90% over six orders of magnitude of *P. parasitica* DNA concentrations with a linear relationship between the log of the DNA concentration and the Cq value with a coefficient of determination $R2 = 0.9993$ and $P < 10^{-6}$. Using these conditions, the lowest *P. parasitica* DNA concentration that was accurately and reproducibly detected by PCR was 130 fg.μL-1, i.e., 260 fg of DNA. These real-time PCR conditions were selected to test the substrate.

Optimization and validation of the real-time PCR assay for DNA extracted from substrates

The optimal real-time PCR conditions for accurate quantification of *P. parasitica* in *Choisya* substrate were validated as follow. Two 10-fold serial dilutions of DNA were analyzed: dilutions of *P. parasitica* DNA, supplemented (condition 1) or not (condition 2) with DNAs purified from substrate matrix and previously characterized as lacking *Phytophthora* DNA (data not shown). The Ypt1 copy numbers were determined for each dilution. The chosen PCR conditions were those for whom no differences were observed between the ypt1 copy numbers amplified in the two conditions (Fig. 2).

The final optimized real-time PCR reaction mixture consisted of the following: 1 μm of each primer, 0.5 $mg.mL^{-1}$ BSA and 1X LightCycler® 480 DNA SYBR Green I Master mix (Roche, Basel, Switzerland. The chosen conditions for primers amplification were 61 °C for 45 s. Using this real-time PCR protocol, the efficiency consistently exceeded 88% over six orders of magnitude of *P. parasitica* DNA concentrations. This protocol was highly reproducible between sample replicates and users.

Efficiency of different treatments against *Phytophthora* root rot in *C. ternata*

Symptom observation

Disease symptoms appeared only on plants with artificial inoculation of *P. parasitica*. These symptoms were more or less severe depending on the tested treatments (Fig. 3). In the case of no treatment T1i, 33% of the plants were healthy (h), 50% with moderated symptoms (m) and 17% with severe symptoms (s). Chemical treatment T2i was apparently the most effective treatment capable of reducing symptoms, with 67% (h), 33% (m) and 0% (s). Treatment modules Ci, Di, Ei, Fi and Gi were seemingly not able to reduce the symptom severity. Furthermore, treatment module Hi led presumably to increased symptoms, with 64% (s) and less than 10% (h). On the contrary, the biological treatments Ai and Bi visibly reduced symptoms. These two modules showed fewer symptoms than the no treatment module (T1i) with 42% and 33% (h), 50% and 67% (m) and 8% and 0% (s), respectively. The BCA-treated plants that were not inoculated with *Phytophthora* showed no disease symptoms and exhibited similar plant growth compared to the untreated plants (data not shown).

Effects of the tested treatments on pathogen density in the substrate

Quantification of the *P. parasitica* density in the plant substrate for all *Phytophthora* inoculated modalities was performed using the previously developed real-time PCR assay. The density of the quantified ypt1 was different depending on the treatment (Fig. 4). In the T1i control, the pathogen density was 8.2×10^5 *ypt1* copy numbers g^{-1} dry substrate. The lowest *ypt1* copy numbers g^{-1} dry substrate were observed for T2i, for which the *Phytophthora* density was significantly

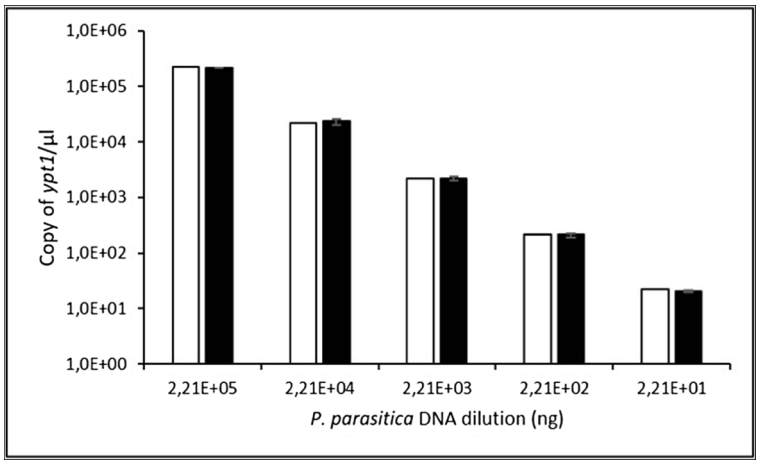

Fig. 2 Quantification of *P. parasitica* in substrate using qPCR to target the gene *Ypt*1. White columns indicate the number of *ypt1* copy's in the pure *P. parasitica* DNA and black columns the number of *ypt1* copy's in the mix *P. parasite* and substrate DNA. Bars plot means ± SD of three replicates

reduced to 1.91×10^5 *ypt1* copy numbers g^{-1} dry substrate. The pathogen density for treatments Ci, Di, Ei, Fi and Gi (respectively, 7.06×10^5, 7.83×10^5, 7.89×10^5, 6.16×10^5 and 6.48×10^5) was not significantly different from T1i. Treatment Hi showed a significant increase in the density of *Phytophthora* in the substrate with 1.33×10^6 *ypt1* copy numbers g^{-1} dry substrate. Modules Ai and Bi led to a significant reduction of pathogen development in the substrate compared to T1i with, respectively, 3.68×10^5 and 3.06×10^5 *ypt1* copy numbers g^{-1} dry substrate.

Correlation between symptom observation and pathogen density

The symptom severity and *ypt1* copy number g^{-1} dry substrate varied widely between the different modules. To understand the potential link between the *Phytophthora* density in the substrate and the symptom severity, correlation tests were performed considering all modalities (Fig. 5). A high and significant correlation ($\rho = 0.77$, with a p-value <0.05) between *ypt1* copy number g^{-1} dry substrate and *C. ternata* death (d) was highlighted.

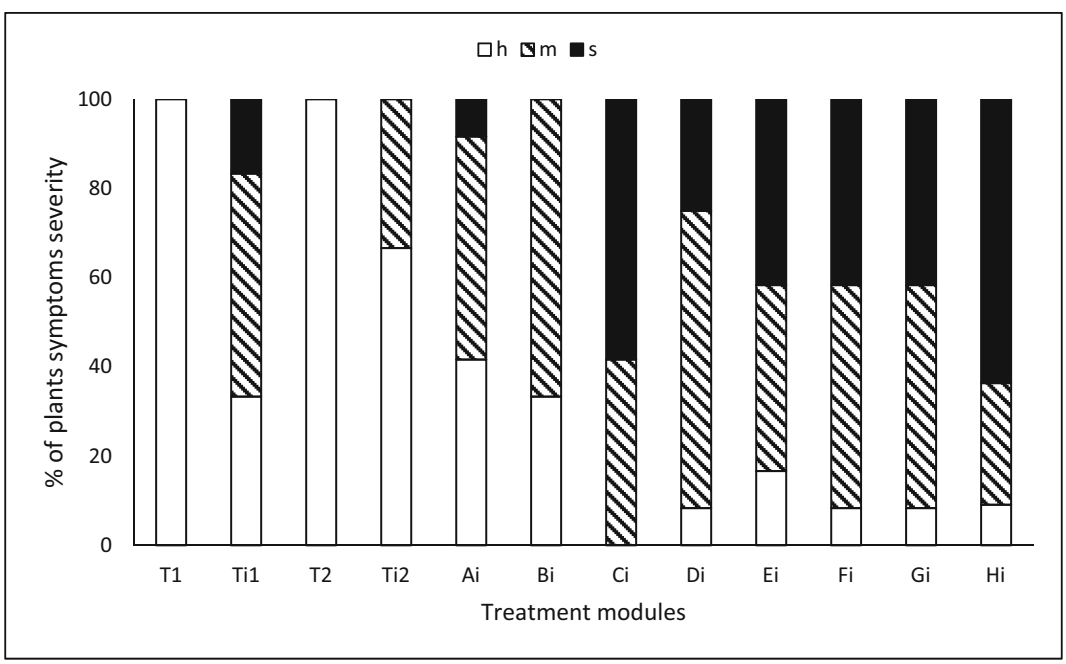

Fig. 3 Percentage of symptoms severity with each treatments modules after 1 month of inoculation. (h) healthy plant, (m) plants with moderate symptoms and (s) plants with severe symptoms. (i) inoculated with *P. parasitica*

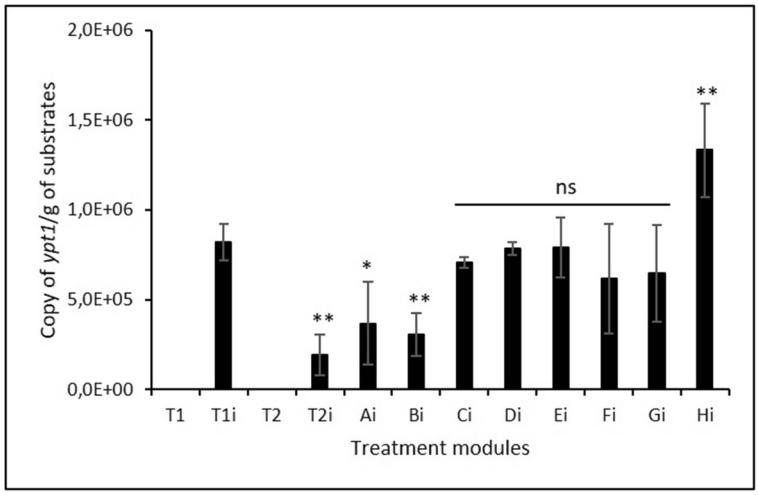

Fig. 4 Quantification of *P. parasitica* development in the substrate with different treatment modules by quantification of *Ypt*1 gene. Significance (Dunnett's post multiple comparison test after ANOVA): $*p < 0.05$, $**p < 0.005$, vs the no treated (T1i) control group. (i) inoculated with *P. parasitica*, bars plot means ± SD of three replicates

Discussion

Pathogenic *Phytophthora* species of *C. ternata*

In this study, *P. parasitica* and *P. tropicalis* were isolated from the roots of infected *C. ternata*. *P. parasitica* has a wide host range of more than 900 ornamental plants, agricultural plants and trees (Farr and Rossman 2017), and it is already known to infect *C. ternata* cultures, leading to large economic losses (Talbot and Wedgwood 2009; Farr and Rossman 2017). Other *Phytophthora* spp., such as *P. citricola*, *P. cryptogea*, *P. cinnamomi* and *P. citrophthora*, were also previously isolated from *C. ternata* in 2008 by the RHS (Talbot and Wedgwood 2009). However, the method used to identify the different isolated strain was not precisely described. Furthermore, morphological differentiation within the genus *Phytophthora* requires substantial experience of the manipulator and is a labor-intensive process (Drenth et al. 2006). This identification technique remains difficult and may lead to some confusion between *Phytophthora* species because of interspecific overlaps and the intraspecific plasticity of diagnostic characteristics (Kong et al. 2004). The second species isolated herein on

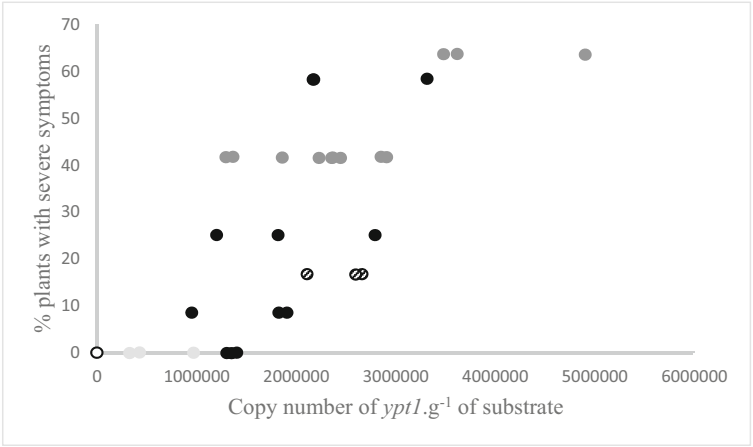

Fig. 5 Correlation between *C. ternata ypt1* copy number in 1 g of substrate and the percentage of plants with severe symptoms. White dots represent non inoculated plants (non treated T1 and treated with Mefenoxam T2, with no detection of *P. parasitica* and no symptoms), white dots with stripes represents inoculated but not treated plants Ti1, light grey dots represents inoculated plants treated with Mefenoxam Ti2, dark grey dots represents inoculated plants treated with one BCA (Di, Ei, Fi, Gi), black dots represents inoculated plants treated with two BCAs (Ai, Bi, Ci, Hi)

C. ternata was *P. tropicalis*. This species has been reported in the Pacific islands, North America and Europe (Farr and Rossman 2017). Therefore, its full geographic distribution is unclear (Aragaki and Uchida 2001; Zhang et al. 2004; Farr and Rossman 2017). The host range of *P. tropicalis* contains more than 30 plants (Farr and Rossman 2017), such as *Atharanthus roseus* (Hao et al. 2010), *Cuphea ignea* (Cacciola et al. 2006), *Cyclamen persicum* (Gerlach and Schubert 2001), and *Rhododendron* spp. (Hong et al. 2006; Galli et al. 2013). To our knowledge, this is the first report of the isolation of *P. tropicalis* from *C. ternata* roots. This species could originate from other exotic plants by cross-contamination in the station. It would be interesting to evaluate *P. tropicalis* infection, colonization and virulence on *C. ternata* in comparison to the more classically described pathogens on this plant with *P. parasitica*. Other oomycetes detected on *C. ternata* belonged to the *Pythium* genus. The isolation technique utilized the oomycete target, which explained why different oomycete genera could be isolated with *Phytophthora*. RHS also isolated *Pythium* species from *C. ternata.* RHS reports that both pathogens could be found on the same plants and could cause root rot as a pathogenic consortium. The similar symptoms induced by these pathogens make it difficult for horticulturists to identify the pathogen species (Talbot and Wedgwood 2009), and thus accurate microbial identification is essential for pathogen inspection and survey (Kong et al. 2004). A larger sample of symptomatic plants could be assessed in the future by horticulturists and nurserymen to constitute a broader strain voucher.

Real-time PCR assay validation

Our results showed that different species of *Phytophthora* can be pathogenic to *C. ternata* as previously described (Talbot and Wedgwood 2009). This observation highlights the importance of a diagnostic tool capable of the simultaneous detection of different *Phytophthora* species in a single run. Here, genus-specific PCR primers were used. *P. parasitica* and *P. tropicalis* were not in the list of the tested 35 *Phytophthora* species (Schena et al. 2006). A positive amplification was observed for these two species, with an efficiency exceeding 90%. A second amplicon with a Tm close to the targeted amplicon was observed for *P. parasitica* isolated from *C. ternata* and *P. parasitica* INRA-310 strain but not for *P. tropicalis*. This second amplicon could be due to the existence of two different alleles for the *ypt1* gene, although no such report could be found in the literature. As the amplified genomic sequence contains an intron, this second amplicon could also be an intronic variant in the diploid genome of the used strain of *P. parasitica*. The intensity of the second amplicon was only minor in comparison to the targeted amplicon. Due to their acceptable specificity and good efficiency, YPh1F and YPh2R primer set were selected to evaluate the density of *Phytophthora* in *Choisya* substrate. The developed SYBR Green PCR assay allowed us to quantify as few as 3 pg of *Phytophthora* isolate DNA, close to the threshold observed by Schena et al. 2006. A higher threshold could be expected when targeting multi-copy genes (Hayden et al. 2004; Ippolito et al. 2004; Schena and Cooke 2006). In the present analysis, none of the tested PCR conditions with primers targeting multi-copy genes led to an acceptable PCR efficiency.

Specific techniques for *Phytophthora* inoculation

To produce a sufficient quantity of *Phytophthora* inoculum for the experiment, different techniques were explored (data not shown). The use of zoospores to inoculate the plants was considered in the case of small-scale test in the laboratory. However, production of a sufficient inoculum and its transport to the experimental station without affecting the viability of the zoospores was impossible. Another possibility was to use *Phytophthora* oospores. Oospores are produced by oomycete sexual reproduction. *P. parasitica* is a heterothallic organism that requires two compatible mating types, A1 and A2, for oospore production (Ko 1978; Meng et al. 2014). However, this method of reproduction is very slow and generally insufficient. The method chosen for *Phytophthora* inoculum production on rice grains, as described by (Holmes and Benson 1994; Loyd et al. 2014), is a known, very effective and rapid technique to produce a sufficient inoculum for the artificial contamination of plants in the experimental station.

Validation of the real-time PCR method in the substrate matrix

The ability to detect and quantify the density of *Phytophthora* species in the substrate is of major importance, for example, to evaluate substrate contamination prior to use or to monitor horticultural facilities and plant production. The real-time PCR assay can also be used as a diagnostic approach to evaluate the capacity of

some BCAs to reduce *Phytophthora* development in the plant substrate.

However, use of the real-time PCR technique to evaluate the concentration of *Phytophthora* in total DNA extracted from the substrate could lead to an underestimation of the pathogen density, and possibly even false negative results. Total DNA samples extracted from substrate can be contaminated by many PCR inhibitors, such as humic acids, tannins and lignin-associated compounds (Cullen and Hirsch 1998; Bridge and Spooner 2001; Matheson et al. 2010). Using an adapted method to extract substrate DNA is the first essential step to reduce this contamination. In the present study, the Fast DNA spin Kit for Soil (MP-Biomedicals, Santa Ana, CA, USA) provides high-quality DNA in sufficient quantities. To validate the experimental conditions used for real-time PCR in the substrate, prior assessment of the accurate quantification of the targeted gene is essential. Here, we used the approach of mixing the *Phytophthora*-free substrate DNA extract with 10-fold serial dilutions of *Phytophthora* isolate DNA extract, and then compared the quantification results to the same 10-fold serial dilutions of *Phytophthora* isolate DNA. For the final chosen PCR conditions, the amplification and efficiency of the real-time PCR assay were not affected by the presence of co-extracted substrate contaminants. The method was thus validated for use in further analyses.

Efficiency of the tested treatments against *Phytophthora*

The severity of the observed *Phytophthora* symptoms on *C. ternata* and the density in the substrate were significantly correlated. *Phytophthora* detection and quantification in the substrate were indicative of the disease severity on the plants. This link has now been further studied and defined as a possible predictor for monitoring plant production.

The survival rate of *C. ternata* was found herein to be treatment-dependent. All the tested solutions (chemical or biological) have been previously commercialized to limit the impact of *Phytophthora* in the ornamental industry. The tested individual BCA agents had been chosen according to previous promising results on *C. ternata* from Astredhor (Cannesan 2017, internal communication). BCAs had been here combined to have uncommon dual mix: bacteria with filamentous or mycorrhizal fungi and filamentous with mycorrhizal fungi. Some studies have demonstrated the possible great potential of using BCAs to limit *Phytophthora* development (Mcquilken et al. 2001; Brunner et al. 2005; Zheng et al. 2005). In the present study, chemical treatment remained the optimal technique to achieve the best survival rate. However, this treatment is not a sustainable option due to the increasing resistance of *Phytophthora* to this chemical and environmental concerns necessitating a reduction of the use of fungicides (Gullino and Kuijpers 1994). The second best survival rate observed with biological treatment modules was observed for the combined treatments with mycorrhiza and a filamentous fungus: *G. catenulatum* with *G. intraradices* (Ai) and *G. intraradices* with *T. atroviridae* (Bi). Other studies have demonstrated the advantage of combining biological treatments, and their use is increasingly being considered (Roberts et al. 2005; Thilagavathi et al. 2007; Chemeltorit et al. 2017). This approach aims to use the potential synergistic effects of organisms, which is essential for BCA treatment, to assure an effective and efficient impact (Spadaro and Gullino 2005). To our knowledge, no other study has reported the capacity of these two combined treatments to reduce the development of *P. parasitica* in substrate and to protect *C. ternata* against this pathogen. Other studies have demonstrated that the combination of fungi and bacterial species, respectively, *Trichoderma hamatum* and *Pseudomonas aeruginosa*, is able to significantly reduce the incidence of *P. capsici* disease in chili pepper seedlings (Chemeltorit et al. 2017). In our study in contrast, the combined use of bacteria and fungi in module Ci (*B. amyloliquefaciens* and *G. intraradices*) showed no significant reduction. The effect of the combined application of BCA can greatly vary (Xu et al. 2011) according to the host plant (Schisler et al. 1997). Indeed, the added bacteria may be unable to survive in the rhizosphere due to various factors, such as acclimatization to the local environment and competition with native strains. These difficulties are more prevalent when the bacterial strain used is derived from a commercial origin and is not native to the rhizosphere of *C. ternata* (Lee et al. 2015). Concerning the combined application of module Hi (*G. catenulatum* and *B. amyloliquefaciens*), this treatment increased disease severity. In our analysis, the activity of the BCA could be affected by possible competition between the added microorganisms. It is possible that *B. amyloliquefaciens* and *G. catenulatum* participated in a competitive interaction that allowed the development of *P. parasitica.* (Bora et al. 2004) showed that the combined application of *Pseudomonas putida*

strains 30 and 180 resulted in lower suppression of *F. oxysporum f. sp. melonis* in comparison to treatment with only strain 30. (Guetsky et al. 2001) showed that the improved efficacy of the combined treatment is maximized when microorganisms have different modes of action. Finally, the 4 treatment modules (D, E, F and G) containing each only one species of BCA did not significantly reduce disease symptoms. In the case of *C. ternata*, the protection capacity of BCA appeared to be improved for the chosen combination of BCAs, probably because of a synergic effect between the microorganisms. However, this synergistic protection seems to be BCA species-dependent.

However, it could not be excluded that the absence of observed efficiency of some BCA microorganisms results from their incapacity to colonize and survive in the substrate. Monitoring their installation after inoculation, with specific real-time PCR assay, could be a valuable asset to optimize their use for plant protection. It could also be interesting to test their survival rate and development in different culture conditions to favor their implementation. Furthermore, only mix of 2 BCAs had been tested on this work. Based on all of the above results, it could also be interested to test mix of 3 BCAs and possible synergic effect. Relevance of treatments has been here evaluated only in the case of *P. parasitica* inoculation. It cannot be excluded from this study that the other characterized oomycetes could be pathogens on *C. ternata* in nurseries, particularly a consortium of the 2 pathogens *Phytophthora* and *Pythium.* Efficiency of all BCA treatments could also be tested for infestation with other *Phytophthora* species (such as *P. tropicalis*), or even other oomycetes like *Pythium.*

Conclusion

C. ternata cultures are sensitive to *Phytophthora* infection. *P. parasitica* is one of the major *C. ternata* pathogens in France. However, *P. tropicalis* was reported, herein, for the first time on infected *Choisya*. Culture losses due to this pathogen can inflict serious economic damage to plant nurseries. Chemical treatment remains the most effective and used approach to limit the damage caused by *Phytophthora* on *C. ternata* cultures. However, the desire to reduce the use of fungicides and increasing resistance of *Phytophthora* to these products highlight the necessity of identifying alternative and sustainable solutions. The most promising approach is the use of BCA to limit the development of pathogens in the substrate. In this study, a validated real-time PCR assay for *Phytophthora* detection and quantification in a substrate was successfully used to monitor the development of *Phytophthora* after BCA addition during *C. ternata* cultivation. Application of different combined treatments of *G. catenulatum* and *G. intraradices* or *G. intraradices* and *T. atroviridae* showed an interesting decrease in disease symptoms and pathogen development, similar to those observed with chemical treatment. This increased effect of BCA could be explained by the potential synergistic effects of the organisms. However, other combined BCAs can, conversely, increase the development of *P. parasitica* due to potential competition between the BCAs. Finally, other BCA treatments had no effect on the pathogen. The use of biological solutions is very promising. More research is needed to understand the underlying mechanisms of action and to optimize conditions for their competitive development in the rhizosphere for their effective use as a replacement for chemical treatment.

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Compliance with ethical standards Authors declare that:

- This manuscript has not been submitted to more than one journal for simultaneous consideration.
- This manuscript has not been published previously (partly or in full).
- This paper relate a single study and has not been split up into several parts.
- No data, including images, have been fabricated or manipulated to support our conclusions.
- No data or text by others are presented as if they were the author's own.
- Consent to submit has been received explicitly from all co-authors, as well as from the responsible authorities - tacitly or explicitly - at the institute/organization where the work has been carried out, **before** the work is submitted.
- Authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.
- Authors group, corresponding author, and order of authors have been validate by both authors and responsible authorities before submission of this manuscript.
- Upon request, we are prepared to send relevant documentation or data in order to verify the validity of the results.

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