RESEARCH PAPERS

Biological control of strawberry soil-borne pathogens *Macrophomina phaseolina* and *Fusarium solani*, using *Trichoderma asperellum* and *Bacillus* spp.

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Summary. In south-western Spain, *Macrophomina phaseolina* and *Fusarium solani* were found to be associated in strawberry plants with, respectively, charcoal rot, and crown and root rot symptoms. For management of both fungal diseases, the antagonistic effects of two commercial formulations, one based on *Trichoderma asperellum* T18 strain (Prodigy®) and the other on *Bacillus megaterium* and *B. laterosporus* (Fusbact®), were evaluated *in vitro* and under controlled environment and field conditions. Two inoculation methods (root-dipping and soil application) and two application times (pre- and post-pathogen inoculation, as preventive and curative treatments, respectively) were assessed. Dual plate confrontation experiments demonstrated the antagonistic effects of *T. asperellum* and *Bacillus* spp. by inhibiting radial growth of *M. phaseolina* and *F. solani* by more than 36%. Preventive application of *T. asperellum* by root-dipping reduced the incidence of charcoal rot (up to 44% in a growth chamber and up to 65% under field conditions) and also reduced disease progression, the percentage of crown necrosis, as well as the level of infection measured as ng of pathogen DNA g^{-1} plant by quantitative real-time PCR. This treatment was also the most effective for reduction of crown and root rot caused by *F. solani* (up to 100% in a greenhouse and up to 81% under field conditions). These results were nearly comparable with the control achieved using chemical fungicides. The *Bacillus* spp.-based formulation was also effective for control of charcoal rot and showed variable results for control of *F. solani*, depending on the growth conditions.

Key words: biocontrol agents, charcoal rot, crown and root rot, *Fragaria × ananassa* Duch.

Introduction

Strawberry (*Fragaria × ananassa* Duch.) is an important berry crop in Spain. Cultivation of strawberry is mainly dependent on the use of chemical fumigants for soil disinfestation. The phaseout of the majority of soil fumigants, especially methyl bromide (MB) in compliance of the Montreal Protocol, has been identified as the main cause for the emergence and re-emergence of soil-borne pathogens affecting strawberry production, causing substantial economic losses and costly control measures.

In recent years, the soil-borne pathogenic fungi *Macrophomina phaseolina* (Avilés *et al.*, 2008), and *Fusarium solani* (Pastrana *et al.*, 2014) have emerged in strawberry crops at Huelva (south-western Spain) as the causal agent of charcoal rot and crown and root rot, respectively. *Macrophomina phaseolina* is a sclerotial fungus with a broad host range. It has also been reported affecting strawberry in other countries (Benlioğlu *et al.*, 2004; Mertely *et al.*, 2005 ; Zveibil and Freeman, 2005; Tjamos *et al.*, 2006; Golzar *et al.*, 2007; Koike, 2008; Baino *et al.*, 2011; Sharifi and Mahdavi, 2011; Sánchez *et al.*, 2013). In Spain, the incidence of *M. phaseolina* has increased over the past several years in Huelva province (Bascón, 2009). The number of affected plants increases throughout the

season, coinciding with high temperatures and water deficits. *Fusarium solani* is a complex (*Fusarium solani* species complex, FSSC) comprised of about 50 phylogenetic species (Zhang *et al.*, 2006; O'Donnell *et al.*, 2008). Species associated with the FSSC are ubiquitous and can be found as soil-inhabiting saprophytes, rhizosphere colonizers, or causing diseases in many plant species or opportunistic infections in humans (Booth, 1971).

The control of both diseases in strawberry is challenging because the causal pathogens, *M. phaseolina* and *F. solani*, can survive for long periods in soil as resistant structures (respectively, sclerotia and chlamydospores), and both are disseminated by various means, including wind, soil and infected plant material. Chemicals currently available for pre-plant soil disinfestation are not effective for the eradication of *M. phaseolina* (Zveibil *et al.*, 2012; Chamorro *et al.*, 2015), and their efficacy in treatment of *F. solani* is as yet unknown. In addition, the prohibition of most of the chemical fungicides by the EU (Directive 91/414/CEE) and adverse effects of their indiscriminate use on the environment and human health, have promoted research in the field of biological control of plant diseases as an effective and sustainable alternative.

Biological control agents in the genera of *Trichoderma* and *Bacillus* have been extensively used for the control of plant diseases (Cawoy *et al.*, 2011; Naher *et al.*, 2014). The mode of action of these antagonistic organisms includes mycoparasitism, production of antibiotics and secondary metabolites, competition for space and nutrients, and induction of defense responses including systemic resistance responses in the plant (Howell, 2003; Benítez *et al.*, 2004; Compant *et al.*, 2005). Also, they are able to enhance growth of plants and increase crop yields (Sturz *et al.*, 2000; Welbaum, *et al.*, 2004; Harman, 2006).

The objective of the present study was to evaluate the efficacy of two commercial formulations, one based on *Trichoderma asperellum* and the other on two species of *Bacillus,* for control of strawberry diseases caused by *M. phaseolina* and *F. solani*. Experiments were performed *in vitro,* and *in vivo* under controlled environment and field conditions.

Materials and methods

Fungal pathogens and plant material

Macrophomina phaseolina isolate TOR-99 and *F. solani* isolate FPOST-81 were obtained from symp-

tomatic strawberry plants and conserved as single spore isolates at the IFAPA Las Torres-Tomejil collection (Sevilla, Spain). Growing conditions for *M. phaseolina* were 28°C for 7 to 10 d in darkness on potato dextrose agar (PDA; Difco). Growing conditions for *F. solani* were 25°C for 2 d in darkness and 5 d in 12 h photoperiod on PDA.

One-month-old strawberry plants cv. Fortuna for *M. phaseolina* and cv. Camarosa for *F. solani* were used in growth chamber and greenhouse experiments and field trials. These cultivars were selected for their respective susceptibilities to *M. phaseolina* (Chamorro *et al.*, 2014) and *F. solani* (Pastrana *et al.*, 2014).

Biological control agents and chemical products

Two biocontrol agents (BCAs) were tested for efficacy: Prodigy® (AMC Chemical) composed mainly of *Trichoderma asperellum* T18 strain (10^8 cfu g^{-1}) and presented as a wettable powder, and Fusbact® (AMC Chemical) composed of *Bacillus megaterium* B157 and *B. laterosporus* B197 (hereafter referred to as *Bacillus* spp.) at a concentration of 10^7 cfu mL^{-1} in a liquid formulation. Two chemical fungicides were used for comparison of their efficacy with the tested BCAs: methyl thiophanate-70% (w/w) for the control of *M. phaseolina,* and carbendazim-50% for the control of *F. solani*.

In vitro tests

Dual plate confrontation experiments (Royse and Ries, 1978) were carried out to evaluate the *in vitro* antagonism of *T. asperellum* T18 strain against *M. phaseolina* TOR-99 and *F. solani* FPOST-81. A 5-d-old mycelial disc (5 mm diam.) from the margin of the colony of the fungal pathogens was placed at the periphery of each of five replicate PDA plates (90 mm) and incubated for 2 d at 25°C. A mycelial disc of *T. asperellum* strain T18 was then placed 5 cm from the inoculum point of the pathogen and the plates were incubated for an additional 7 d at 25°C. The growth (mm) of the pathogen was recorded between the inoculation point and farthest point of the colony (R1) and between the inoculation point and the border in which the pathogen and *Trichoderma* mycelia came into contact (R2). Inhibition of the pathogen development was assessed by calculating the percentage of inhibition of radial growth as *% RGI = [(R1 − R2) / R1] × 100* (Royse and Ries, 1978). The profusion

of growth over opposite microorganisms (*i.e.*, over-growth) was examined.

For evaluation of antagonism caused by *Bacillus*, 5-d-old mycelial discs (5 mm diam.) from the margin of the colonies of test pathogens were placed on the center of each of five replicate PDA plates (90 mm). Bacterial suspensions (10^7 cfu mL^{-1}) (50 μL) were equidistantly placed, from the inoculum point, on the edge of PDA plates 48 h after pathogen inoculation. Plates were incubated at 25ºC for 7 d, and the inhibition of fungal growth was assessed by measuring the percentage of colony growth inhibition (% CGI) calculated as $\%CGI = [(D1 - D2) / D1] \times 100$ where: D1 corresponds to colony diameter of the pathogen in control plates, and D2 represents the colony diameter of the pathogen in treated plates. In both tests, controls were pathogen cultures without

T. asperellum or *Bacillus* spp., respectively. Experiments were repeated five times and results expressed as the mean colony diameter ± standard error of five replicates.

Plant growth chamber and greenhouse experiments

For *M. phaseolina*, two pot experiments were carried out in a growth chamber maintained at 29/26°C and 60/40% relative humidity (day/night) with a daily 16 h photoperiod of fluorescent light for 3 months. For *F. solani*, one pot experiment was carried out in a greenhouse from November to January in environmental conditions with maximum temperature between 28 and 32ºC, minimum temperature between 8 and 10ºC, and irrigation of plants as needed.

Table 1. Biological and chemical treatments assessed for control of charcoal rot (caused by *Macrophomina phaseolina*) and crown and root rot (caused by *Fusarium solani*) in strawberry.

Treatment	Abbreviation	Composition	Time of application[a]	Mode of application	Inoculated pathogen
Biological	TPD	*Trichoderma asperellum* T18	Preventive	Root-dipping	*M. phaseolina* *F. solani*
	BPD	*Bacillus megaterium* B197 + *B. laterosporus* B157	Preventive	Root-dipping	*M. phaseolina* *F. solani*
	TPS	*Trichoderma asperellum* T18	Preventive	Soil application	*M. phaseolina* *F. solani*
	BPS	*Bacillus megaterium* B197 + *B. laterosporus* B157	Preventive	Soil application	*M. phaseolina* *F. solani*
	TCS	*Trichoderma asperellum* T18	Curative	Soil application	*M. phaseolina* *F. solani*
	BCS	*Bacillus megaterium* B197 + *B. laterosporus* B157	Curative	Soil application	*M. phaseolina* *F. solani*
Chemical	CPD	Methyl thiophanate-70%	Preventive	Root-dipping	*M. phaseolina*
		Carbendazim-50%	Preventive	Root-dipping	*F. solani*
	CPS	Methyl thiophanate-70%	Preventive	Soil application	*M. phaseolina*
		Carbendazim-50%	Preventive	Soil application	*F. solani*
	CCS	Methyl thiophanate-70%	Curative	Soil application	*M. phaseolina*
		Carbendazim-50%	Curative	Soil application	*F. solani*
Control	WC	Water control	-	Root-dipping and soil application	*M. phaseolina* *F. solani*

[a] Preventive: applied 5 d before inoculation with the pathogen; curative: applied 5 d after inoculation with the pathogen.

Experimental design and treatments

For both pathogens, the experimental designs were randomized complete blocks with nine treatments, including a non-treated water control (Table 1), with eight replicates per treatment (four blocks with two plants per block). For all treatments, two plants per block were included that were not inoculated with the pathogens (non-inoculated controls). Preventive treatments were applied 5 d before inoculation with the pathogen in two ways: root-dipping or soil application. Root-dipping treatments were performed by submerging roots and crowns of the strawberry plants in a suspension of 1% *T. asperellum* T18 formulation or 1% *Bacillus* spp. formulation for 30 min and then transplanting into individual pots (16 cm diam.) containing sterile peat. Soil application was accomplished by pouring 100 mL of a suspension of 0.086% *T. asperellum* T18 formulation, 0.45% *Bacillus* spp. formulation, and 0.1% methyl thiophanate-70% (w/w) for the control of *M. phaseolina,* or 0.06% carbendazim-50% for the control of *F. solani* per plant, at the base of each potted plant. Curative treatments were applied to potted plants in the same manner, but at 5 d after inoculation with the respective pathogens.

Inoculation with the fungal pathogens

Macrophomina phaseolina was grown on PDA plates at 28°C in the dark for 15 d. A suspension was prepared by blending infested PDA with 100 mL of sterile distilled water per plate. Plants grown in 16 cm diam. pots were inoculated by pouring 50 mL of a 1×10^5 microsclerotia mL^{-1} suspension of *M. phaseolina* at the base of each potted plant (Avilés *et al.*, 2009).

Fusarium solani was grown on PDA for 7 d and then 5 mm diam. agar disks from the margin of the colony were transferred to PDB (Potato Dextrose Broth) and incubated for 10 d at 25°C and 150 rpm in a 12-h photoperiod. The spore suspension was filtered through four layers of sterile cheesecloth, spore concentration was determined using a haemocytometer, and the suspension was diluted with sterile water to obtain a final concentration of 1×10^7 conidia mL^{-1} (Pastrana *et al.*, 2014). Each potted plant was inoculated at the base by drenching 100 mL of conidial suspension.

Plants were watered as needed and fertilized with 0.3 g Osmocote® 15 d after transplanting.

Disease assessments

The following parameters were measured: i) incidence of the respective disease, as percentage of dead plants at the end of the experiment, ii) progression of the disease as area under the disease progress curve (AUDPC, Madden *et al.*, 2007) evaluated weekly throughout 3 months, iii) severity of the disease as percentage of crown discoloration, and iv) quantification of the pathogen in symptomatic and dead plants by quantitative PCR (qPCR). The last two parameters were evaluated in plants inoculated with *M. phaseolina* in the second pot experiment.

Isolation of Macrophomina phaseolina and Fusarium solani from inoculated plants

To confirm the cause of plant death, symptomatic strawberry plants were analyzed by plate culture. Plants were surface disinfested in 1% sodium hypochlorite for 2 min and cut longitudinally into two halves. Small disinfested tissues from crowns and roots from one half of each plant were transferred to PDA plates and incubated for 7 d at 25°C in a 12 h photoperiod.

Quantification of Macrophomina phaseolina infection by real-time PCR

The other half of each *M. phaseolina* inoculated plant was homogenized into individual extraction bags (BIOREBA®) in the presence of 1:20 (w:v) PBS buffer, pH 7.2 supplemented with 2% (w:v) polyvinylpyrrolidone (PVP-40) and 0.2% (w:v) sodium diethyl dithiocarbamate (DIECA). Plant crude extracts were subsequently incubated 10 min at 95°C and frozen at -80°C until used. For real-time PCR reactions, 5 μL of 1:20 (v:v) dilutions of the plant crude extracts in sterile distilled water were directly used as samples. PCR reactions were performed in a 20 mL final volume, in 96-well plates in a Chromo4™ thermocycler (Bio-Rad). The reaction cocktail contained 1× SensiMix (SensiMix™ Probe Kit, Bioline), 900 nM each of forward and reverse primers, 150 nM 3'MGB-TaqMan probe labelled with 6'FAM fluorescein (Babu *et al.*, 2011), and 5 μL of plant crude extract dilution. Amplifications were carried out at 95°C for 10 min, and 45 cycles of 15 s at 95°C and 30 s at 60°C. To discard false negative amplifications, an internal positive control consisted of 0.01 ng of λ bacteriophage DNA added to the samples and amplified in parallel PCR reactions using primers λ-F (5'-GGTGGAAACCGCATTCTGTAC-3') and λ-R (5'-CCGTCGAGAATACTGGCAATTT-3') and SYBR Green chemistry, with the amplification conditions described above. For quantitative detection of the

pathogen in plant samples, a standard curve was constructed using 10-fold serial dilutions of genomic DNA from the *M. phaseolina* TOR-99 isolate diluted in healthy strawberry plant extract. Two biological replicates (two samples per plant) and three technical (PCR) replicates of standards, samples and DNA template-free controls were used. DNA concentration was obtained by extrapolation of cycle threshold value through MJ Opticon Monitor software 3.1 (Bio-Rad). The default threshold set by the machine was slightly adjusted above the background to the linear part of the growth curve. To determine the agreement between plate culture and qPCR detection methods for *M. phaseolina* detection, Cohen's kappa index was calculated (Landis and Koch, 1977).

Field trials

Experimental design

Field trials were performed for control of *M. phaseolina* and *F. solani* following the typical strawberry crop cycle and management in south-western Spain, from October to May, in a macro-tunnel covered with 200 μm polyethylene plastic. As Spanish authorities do not allow the application of long survival fungal pathogens to open ground, tight containers with similar dimensions to strawberry beds (30 × 45 × 85 cm) with 100 L of sand soil, previously disinfested with chloropicrin (5kg ha^{-1}, applied by drip irrigation under polyethylene plastic for 20 d), were established for plant growth. Experiments were set up in a randomized complete block design with four blocks (containers) each with eight plants, for the following treatments: TPD, BPD, TCS, BCS, CPD, CCS and WC (see Table 1). For all treatments, eight plants per block were included that were not inoculated with a pathogen (non-inoculated controls). Preventive treatments were applied by root-dipping as previously described, and curative treatments were applied by soil application just when the first disease symptoms appeared (March) at 1% *T. asperellum* formulation, 1% *Bacillus* spp. formulation, 0.1% methyl thiophanate-70% for *M. phaseolina* or 0.06% de carbendazim-50% for *F. solani*. Additional applications of curative treatments were made 7 and 14 d after the first treatment.

Artificial infestation of soil

Prior to infestation, soil was fertilized with NPK Osmocote®-Pro (Everris) (4 kg m^{-3}). *Macrophomina*

phaseolina TOR-99 inoculum was prepared as described above. A suspension of 5×10^4 microsclerotia mL^{-1} was mixed with previously sterilized sand soil 10:100 (v:w) in closed containers to a depth of 20 cm, achieving a final concentration of 5×10^6 microesclerotia kg^{-1}. For *F. solani* isolate FPOST-81, a conidial suspension (4×10^7 conidia mL^{-1}) was thoroughly mixed with sterilized talcum powder (1:1) and air dried to allow the formation of chlamydospores. The number of *F. solani* propagules in the infested powder was determined by dilution plating. The inoculum was mixed with the sand soil along two lines per container at 20 cm depth using 100 g of infested powder (5×10^6 cfu g^{-1}) per 1700 g of soil, getting a final concentration of 2.9×10^8 cfu kg^{-1}. Strawberry plants were planted 7 d after soil infestation. Plants were watered by drip irrigation as needed.

Disease assessments

The following parameters were evaluated: i) incidence of the disease, as percentage of dead plants at the end of the trial; ii) AUDPC evaluated weekly throughout 7 months; iii) percentage of crown discoloration (only in the case of *M. phaseolina*); and iv) crop yield measured as weight (g) of fruit produced per plant.

Statistical analyses

Experiment by treatment interactions were calculated for the two pots experiments for control of *M. phaseolina*. As no significant interactions were observed ($P \geq 0.05$), incidence and AUDPC results are reported as the average of combined analyses (Table 2). Percentage data were transformed (arcsine $\sqrt{Y/100}$) and analysis of variance (ANOVA) was used to test for statistically significant effects of independent variables. Where significant F values were obtained, means were compared using Fisher's LSD test at $P \leq 0.05$. All statistical analyses were performed with Statistix 9.0.

Results

Antagonism *in vitro*

Dual plate confrontation of *T. asperellum* and the pathogens showed 55% radial growth inhibition (RGI) for *M. phaseolina* and 39% for *F. solani*. Overgrowth of *M. phaseolina* by *T. asperellum* was

Table 2. Means of disease incidence, AUDPC, proportions of crown necrosis and amounts of pathogen DNA in plant tissues, after application of different biological and chemical treatments for control of charcoal rot caused by *Macrophomina phaseolina* in 'Fortuna' strawberry plants grown in growth chamber conditions[a].

Treatment[b]	Incidence (%)[c] (*P*=0.0001)	AUDPC[c] (*P*=0.0001)	Crown necrosis (%)[d] (*P*=0.0016)	ng DNA pathogen/g plant[d] (*P*=0.0013)
WC	100.0 ± 0.0 a	65.7 ± 7.2 a	38.8 ± 3.8 a	39.7 ± 8.7 ab
TPD	56.3 ± 14.7 b	35.7 ± 8.7 bc	9.4 ± 2.1 c	20.1 ± 8.6 bcd
BPD	56.3 ± 14.7 b	24.3 ± 9.1 c	9.0 ± 7.0 c	2.2 ± 1.3 d
TPS	87.5 ± 12.5 a	52.3 ± 8.4 ab	8.3 ± 3.0 c	28.5 ± 7.4 abc
BPS	87.5 ± 8.2 a	50.5 ± 5.8 ab	28.8 ± 7.5 ab	48.7 ± 11.7 a
TCS	87.5 ± 8.2 a	53.8 ± 7.0 ab	11.3 ± 2.1 c	30.2 ± 8.7 abc
BCS	100.0 ± 0.0 a	60.1 ± 7.9 a	14.4 ± 2.1 bc	37.9 ± 8.5 ab
CPS	31.3 ± 9.1 b	18.6 ± 5.8 c	10.3 ± 6.0 c	7.2 ± 2.7 cd
CCS	43.8 ± 11.3 b	20.9 ± 5.5 c	6.4 ± 2.3 c	18.2 ± 8.2 bcd

[a] Growth chamber adjusted at 29/26ºC and 60/40% relative humidity (day/night) and 16 h photoperiod over 3 months.
[b] WC: Water control, plants inoculated with the pathogen and not treated; TPD: *Trichoderma asperellum* T18 preventively applied by root-dipping; BPD: *Bacillus* spp. preventively applied by root-dipping; TPS: *Trichoderma asperellum* T18 preventively applied by soil application; BPS: *Bacillus* spp. preventively applied by soil application; TCS: *Trichoderma asperellum* T18 curatively applied by soil application; BCS: *Bacillus* spp. curatively applied by soil application; CPS: Methyl thiophanate-70% preventively applied by soil application; CCS: Methyl thiophanate-70% curatively applied by soil application.
[c] Values are means ± standard error of two experiments with four replications and two plants per replication. Different letters indicate significant differences among treatments within the same column according to LSD test (*P*≤0.05).
[d] Values are means ± standard error of four replications with two plants per replication. Different letters indicate significant differences among treatments within the same column according to LSD test (*P*≤0.05).

observed, and *T. asperellum* also produced competitive growth, i.e. the antagonist grew faster than the pathogen and gained space and nutrients. No overgrowth of *F. solani* by *T. asperellum* was observed. Dual plate confrontation of *Bacillus* spp. with *M. phaseolina* or *F. solani* showed colony growth inhibition (CGI) of 43% and 37%, respectively.

Biocontrol of charcoal rot caused by *Macrophomina phaseolina*

Growth chamber experiments

In both experiments for control of *M. phaseolina* under growth chamber conditions, all plants inoculated with the pathogen but not treated (water control) died. No symptoms were observed in control plants treated with the biological or chemical products but not inoculated (non-inoculated controls). The preventive application of the BCAs by root-dipping (TPD and BPD) significantly (*P*=0.0001)

reduced the incidence of charcoal rot up to 44%, exhibiting similar levels of reduction to what was obtained with chemical products (Table 2). In the same way, these biological and chemical treatments significantly reduced the AUDPC (*P*=0.0001) (Table 2). Also, the application of any biological treatments (with the exception of BPS) significantly reduced the percentage of crown discolouration compared to the non-treated control, at similar levels to the chemical treatments (Table 2).

Quantification of Macrophomina phaseolina by qPCR

The protocol used here detected *M. phaseolina* in infected strawberry plants without the requirement for DNA extraction. False negatives were rejected due to internal positive control amplified in all samples (data not shown). Limit of detection of *M. phaseolina* was 10 pg of genomic DNA diluted in plant crude extract from a healthy strawberry plant. Efficiency of the PCR reaction was 90.5%. The coefficient of correlation (R^2) of standard curve was 0.998.

The infection level of *M. phaseolina* in the inoculated plants, measured as ng of pathogen DNA g⁻¹ tissue by qPCR, varied among the groups of treated plants. All samples amplified the positive internal control based on λ DNA. BPD and CPS treated plants had an average of 2.2 and 7.2 ng of *M. phaseolina* DNA g⁻¹ of plant tissue, respectively, which was significantly less than untreated control plants (39.7 ng *M. phaseolina* DNA g⁻¹ of plant tissue). The application of TPD and CCS also reduced the infection level in treated plants which contained 20.1 and 18.2 ng pathogen DNA g⁻¹ plant tissue, respectively, but this amount of DNA was not significantly different from the control (Table 2).

Macrophomina phaseolina was isolated from all dead plants, mainly from crown tissues (34% of dead plants), from roots (14%), and from both, crown and root tissues from each dead plant (52%). A substantial correlation (Cohen's kappa index = 0.66 ± 0.10) was detected between results obtained from plate isolation and real-time PCR detection methods.

Field trial

Under field conditions all non-treated strawberry plants that were inoculated with *M. phaseolina* died. The application of the chemical or biological treatments significantly reduced the incidence of charcoal rot compared to the control (*P*=0.0002). Methyl

thiophanate curatively applied, CCS, was the most effective treatment, reducing the incidence of charcoal rot by up to 87%, followed by TPD (65%), and BPD and CPD (50% each). All these treatments also significantly reduced (*P*=0.0103) the AUDPC of charcoal rot. Plants treated by CCS and TPD exhibited the lowest percentage of crown rot (1 and 8%, respectively), both of which were significantly different from untreated control plants. The remaining treatments also gave reductions of crown rot but were not significantly different from the untreated control. Regarding crop yields, plants treated preventively with *T. asperellum* (TPD) or the chemical fungicide (CPD) produced the greatest average weight of fruit per plant (respectively, 58.6 ± 31.4 and 52.2 ± 27.1 g), although these amounts did not differ significantly from the inoculated and non-treated controls (31.2 ± 9.0 g fruit per plant) (Table 3). *Macrophomina phaseolina* was isolated from roots, crowns and petioles from all dead plants.

Biological control of crown and root rot caused by *Fusarium solani*

Greenhouse experiment

In the greenhouse experiment for control of *F. solani*, 50% of inoculated and non-treated (water

Table 3. Means of disease incidence, AUDPC, proportions of crown necrosis and weights of fruit after application of different biological and chemical treatments for control of charcoal rot caused by *Macrophomina phaseolina* in strawberry plants cv. Fortuna under field conditions.

Treatment[a]	Incidence (%)[b] (*P*=0.0002)	AUDPC[b] (*P*=0.0103)	Crown necrosis (%)[b] (*P*=0.0118)	g fruit/plant[b] (NS)
WC	100.0 ± 0.0 a	64.8 ± 12.0 a	62.0 ± 14.3 a	31.2 ± 9.0
TPD	35.4 ± 14.6 cd	23.0 ± 11.9 bc	8.0 ± 4.7 b	58.6 ± 31.4
BPD	50.0 ± 9.0 cd	26.0 ± 4.2 bc	39.2 ± 13.0 ab	32.6 ± 10.8
TCS	66.7 ± 23.5 b	43.0 ± 16.8 ab	38.8 ± 10.1 ab	15.3 ± 6.0
BCS	71.3 ± 10.9 bc	42.8 ± 7.8 ab	36.0 ± 12.7 ab	24.8 ± 4.8
CPD	50.0 ± 0.0 cd	25.3 ± 2.3 bc	48.6 ± 11.5 a	52.2 ± 27.1
CCS	13.4 ± 8.1 d	6.8 ± 4.5 c	1.3 ± 1.2 b	36.6 ± 16.2

[a] WC: Water control, plants inoculated with the pathogen and not treated; TPD: *Trichoderma asperellum* T18 preventively applied by root-dipping; BPD: *Bacillus* spp. preventively applied by root-dipping; TCS: *Trichoderma asperellum* T18 curatively applied by soil application; BCS: *Bacillus* spp. curatively applied by soil application; CPD: chemical fungicide preventively applied by root-dipping; CCS: chemical fungicide curatively applied by soil application.

[b] Values are means ± standard error of four replications with two plants per replication. Different letters indicate significant differences among treatments within the same column according to LSD test (*P*≤0.05). NS: not significant.

control) plants died. No symptoms were observed in control plants treated with the biological or chemical products but not inoculated. The preventive application of *T. asperellum* T18 by root-dipping or soil application (TPD and TPS treatments, respectively) completely prevented incidence of crown and root rot at the same level as that for the preventive or curative application of the chemical fungicide carbendazim (CPS and CCS). The same treatments were effective in the reduction of the disease progress, AUDPC (Table 4). *Bacillus* spp. preventively applied by root-dipping (BPD) also reduced the incidence of dead plants at 50% compared to the non-treated control and reduced the AUDPC, but this difference was not statistically significant (Table 4). *Fusarium solani* was isolated from all symptomatic plants, mainly from roots (67%), but also from crowns (10%) and from

both tissues from individual symptomatic plants (23%). No *F. solani* was recovered from plants that remained alive.

Field trial

In the field trial for control of *F. solani*, 85% of non-treated and inoculated plants were dead at the end of the experiment. All chemical and biological treatments significantly reduced (*P*=0.0001) the incidence of crown and root rot compared to the untreated control. Similarly to the experiment for the control of *M. phaseolina*, the CCS treatment reduced the incidence of the disease (87%), followed by TPD (81%), CPD (80%) and BPD (78%). The application of the BCAs after the appearance of the symptoms (curative treatments) also gave significant reductions in disease incidence, although the efficacy was less (respectively 74% and 59% for TCS and BCS treatments). Consequently, all the biological and chemical control treatments also reduced the AUDPC (*P*=0.0292). The lowest yields, measured as weight of fruit per

Table 4. Means of disease incidence and AUDPC after application of different biological and chemical treatments for control of crown and root rot caused by *Fusarium solani* in 'Camarosa' strawberry plants grown in greenhouse conditions.

Treatment[a]	Incidence (%)[b] (*P*=0.0371)	AUDPC[b] (*P*=0.0419)
WC	50.0 ± 20.2 a	51.5 ± 23.3 a
TPD	0.0 ± 0.0 b	0.0 ± 0.0 b
BPD	25.3 ± 14.3 ab	19.3 ± 11.2 ab
TPS	0.0 ± 0.0 b	0.0 ± 0.0 b
BPS	37.6 ± 23.7 a	17.0 ± 9.8 a
TCS	25.3 ± 14.3 ab	19.3 ± 11.2 ab
BCS	37.6 ± 12.4 a	26.7 ± 9.0 ab
CPS	0.0 ± 0.0 b	0.0 ± 0.0 b
CCS	0.0 ± 0.0 b	0.0 ± 0.0 b

[a] WC: Water control, plants inoculated with the pathogen and not treated; TPD: *Trichoderma asperellum* T18 preventively applied by root-dipping; BPD: *Bacillus* spp. preventively applied by root-dipping; TPS: *Trichoderma asperellum* T18 preventively applied by soil application; BPS: *Bacillus* spp. preventively applied by soil application; TCS: *Trichoderma asperellum* T18 curatively applied by soil application; BCS: *Bacillus* spp. curatively applied by soil application; CPS: Carbendazim-50% preventively applied by soil application; CCS: Carbendazim-50% curatively applied by soil application.
[b] Values are means ± standard error of four replications with two plants per replication. Different letters indicate significant differences among treatments within the same column according to LSD test (*P*≤0.05).

Table 5. Means of disease incidence, AUDPC and weights of fruit after application of different biological and chemical treatments for control of crown and root rot caused by *Fusarium solani* in 'Camarosa' strawberry plants under field conditions.

Treatment[a]	Incidence (%)[b] (*P*=0.0001)	AUDPC[b] (*P*=0.0292)	g fruit/plant[b] (NS)
WC	85.4 ± 8.6 a	31.9 ± 2.8 a	34.4 ± 5.4
TPD	16.1 ± 11.8 b	8.5 ± 7.1 b	51.7 ± 13.7
BPD	18.8 ± 12 b	11.1 ± 9.3 b	99.2 ± 27.2
TCS	22.5 ± 8.9 b	12.3 ± 5.9 b	30.3 ± 5.3
BCS	35.0 ± 7.0 b	13.1 ± 4.2 b	58.4 ± 16.9
CPD	16.7 ± 16.6 b	3.8 ± 3.8 b	83.1 ± 24.4
CCS	11.3 ± 6.5 b	2.4 ± 1.5 b	113.5 ± 42.9

[a] WC: Water control, plants inoculated with the pathogen and not treated; TPD: *Trichoderma asperellum* T18 preventively applied by root-dipping; BPD: *Bacillus* spp. preventively applied by root-dipping; TCS: *Trichoderma asperellum* T18 curatively applied by soil application; BCS: *Bacillus* spp. curatively applied by soil application; CPD: chemical fungicide preventively applied by root-dipping; CCS: chemical fungicide curatively applied by soil application.
[b] Values are means ± standard error of four replications with two plants per replication. Different letters indicate significant differences among treatments within the same column according to LSD test (*P*≤0.05). NS: not significant.

plant, occurred from the control non-treated plants and plants treated with the curative application of *T. asperellum*. The remaining treatments gave greater yields than the control, but these differences were not statistically significant (Table 5). *Fusarium solani* was detected in all dead plants (56% in roots, 40% in crowns and roots, and 4% in roots and petioles).

Discussion

The phaseout of most chemical fumigants used for soil disinfestation has increased the demand for non-chemical alternatives that are effective in controlling diseases caused by soil-borne phytopathogenic fungi. Biological control methods which are economical and free from harmful effects on human and animal health are emerging as effective alternatives to chemical controls. Results derived from the study reported here indicate that *T. asperellum* T18 strain, as the Prodigy® formulation, can reduce the impact of charcoal rot and crown and root rot strawberry diseases caused, respectively, by *M. phaseolina* and *F. solani*. This efficacy has been demonstrated *in vitro*, and in controlled growth and field conditions. The formulation Fusbact®, containing two species of *Bacillus,* also gave effective control of charcoal rot but gave variable results for control of *F. solani* depending on the growth conditions.

Dual plate confrontation experiments demonstrated the antagonistic effects of *T. asperellum* and *Bacillus* spp. by overgrowing or inhibiting radial growth of *M. phaseolina* and *F. solani,* although *T. asperellum* showed greater inhibitory effects on both pathogens than *Bacillus* spp. The antagonistic effects of both BCAs were greater against *M. phaseolina* than against *F. solani*. These results on the efficacy of BCAs *in vitro* are similar to those obtained by other authors reporting efficacy of species of *Trichoderma* and *Bacillus* for the inhibition of *in vitro* growth of soil-borne pathogens including *M. phaseolina* and *F. solani* (Alamri *et al.,* 2012; Hassan *et al.,* 2014; Ramzan *et al.,* 2014), and also with the results obtained in the present study in controlled environment and field conditions.

The efficacy of biocontrol treatments obtained in the experiments performed under controlled conditions varied, depending on the timing and mode of application, with pre-plant root-dipping being the most effective application method. This could easily be incorporated into current strawberry production practices. We recommend the application of BCAs

just prior to transplanting by submerging plant roots and crowns, which can be a site of infection by *M. phaseolina*. The success of preventive root-dip applications could be attributed to the advantage of BCAs in establishing first in the plant rhizospheres and soil which would assist in reducing the opportunity for infection by soil-borne pathogens (Singh *et al.,* 1998; Rini and Sulochana, 2007). Curative treatments were more efficacious with the application of the chemical fungicide under field conditions. This was probably due to the repetitive application of this product once the first disease symptoms appeared (three applications), compared with the single application of preventive treatments. In this way, in growth chamber experiments, the preventive application of *T. asperellum* or *Bacillus* spp. by root-dipping (TPD or BPD treatments, respectively) demonstrated their efficacy in reducing the incidence, the progress of the disease, the level of pathogen infection in the treated plants and the symptomatology of charcoal rot at similar levels to that achieved from the chemical products (CPS and CCS). Furthermore, results under artificially inoculated field conditions were consistent with those observed in controlled environment conditions, as the most effective treatments for the control of *M. phaseolina* in the field were TPD and CCS.

In experiments for the control of *F. solani* in greenhouse conditions, *T. asperellum* T18 applied preventively by root-dipping (TPD) or soil application (TPS), completely prevented the emergence and development of the disease, as did the application of a chemical fungicide. In field conditions, all chemical and biological control treatments reduced the incidence and progress of the disease, although TPD was the most effective treatment against crown and root rot, along with the chemical fungicide carbendazim applied pre- (CPD) or post-inoculation (CCS).

These results on the efficacy of *T. asperellum* as BCA agree with those obtained for control of other strawberry fungal diseases, such as that caused by *Botrytis cinerea* (Kowalska, 2011), and of fungal diseases caused on other crops (Slusarski and Pietr, 2009; Kowalska, 2011, Mbarga *et al.,* 2012). *Trichoderma* spp. have been previously demonstrated as efficient BCAs for the control of *M. phaseolina* in melon, corn, eggplant, sorghum and chickpea (Elad *et al.,* 1986; Ramezani, 2008; Larralde-Corona *et al.,* 2008; Manjunatha *et al.,* 2013), and for the control of *F. solani* in beans, chili and peanuts (Abeysinghe,

2007; Rojo *et al.*, 2007; Naik *et al.*, 2009; Qualhato *et al.*, 2013). To our knowledge, this is the report where the efficacy of *T. asperellum* has been demonstrated for control of *M. phaseolina* and *F. solani* in strawberry.

The efficacy of *Bacillus* spp. was less consistent than that achieved with *T. asperellum* T18, and varied depending on the experimental conditions. The preventive application of *Bacillus* spp. by root-dipping (BPD) reduced the incidence and progress of charcoal rot caused by *M. phaseolina* under controlled and field conditions. In the same way, the application of *Bacillus* spp. significantly reduced the incidence and progress of *F. solani* in the experiment performed in the artificially inoculated field with better results when applied preventively (BPD), but not under greenhouse conditions. *Bacillus megaterium* (Santoyo, 2012), *B. laterosporus* (Saikia *et al.*, 2011) and *Bacillus* spp. (Paulitz and Bélanger, 2001) have been previously demonstrated to provide control of *Fusarium* spp. in several crops. In addition, *B. laterosporus* has demonstrated *in vitro* antifungal activity against *F. solani* (Song *et al.*, 2011) and has potential biocontrol activity against poplar canker disease (Jiang *et al.*, 2015). In strawberry, the effectiveness of treatments by root immersion in a suspension of *B. velezensis* has been previously reported for control of Fusarium wilt caused by *F. oxysporum* f. sp. *fragariae* (Nam *et al.*, 2009). These authors identified colonization of the plant rhizospheres by the BCA as the crucial process for the success of disease control. The variability found in the efficacy of the use of *Bacillus* spp. in the control of strawberry fungal diseases could depend on the environment in which our experiments were performed. There may have been differences in the viability and rhizosphere colonization of *Bacillus* spp. in greenhouse and field conditions, in which different substrate types (peat or sand) were used, and environmental conditions were not the same. These could be the causes of variable efficacy.

Treatments with BCAs have not only reduced the incidence of charcoal rot, but also the symptoms and the amount of *M. phaseolina* present in the infected plants, suggesting that BCAs are limiting the pathogen infection (*e.g.* by the production of hydrolytic enzymes or antibiotics) (Schirmböck *et al.*, 1994; Radheshyam *et al.*, 2012). All treatments (except BPS) gave lower quantities of *M. phaseolina* in plant tissues compared with the non-treated and inoculated water controls. A substantial correlation was found between the two pathogen detection methods used in

this work (plate isolation and quantitative real-time PCR). Quantitative detection could be applied as a reliable method to test the efficacy of BCAs or natural products for control of diseases caused by fungi. Coupled with the high levels of sensitivity and specificity of real-time PCR, the use of crude plant extracts without the need of nucleic acid extraction make this method easy to perform and applicable to high throughout testing.

Under field conditions, no significant differences in weight of strawberry fruit produced per plant were observed with any of the tested treatments. However, the reduction in the number of dead plants by the application of chemical or biological treatments was reflected in an overall enhanced crop yields, but the differences relative to the untreated controls were not statistically significant. Additional field trial studies should be carried out to examine effect of treatments on yields, as the variability of field performance of BCAs is influenced by many factors, including climatic conditions, and differences in the viability and survival of BCAs in the soil (Porras *et al.*, 2009; Kowalska, 2011).

In summary, this study indicates the availability of *T. asperellum* T18 strain (as the main component of the commercial formulate Prodigy®) and to a lesser extent, *B. megaterium* and *B. laterosporus* (as the components of the product Fusbact®), as effective treatments for control of strawberry diseases caused by *M. phaseolina* and *F. solani*. We have also demonstrated a method of application that could potentially improve the efficacy of these biocontrol agents and could be easily incorporated into an integrated management strategy for strawberry production.

Acknowledgements

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