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Control of brown spot of pear by reducing the overwintering inoculum through sanitation

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Abstract *Stemphylium vesicarium*, the causal agent of brown spot of pear, overwinters in the leaf residues of pear and herbaceous plants of the orchard floor. Pseudothecia of the teleomorph, *Pleospora allii*, are formed on these residues where they produce ascospores. New methods were tested aimed at reducing this overwintering inoculum and increasing the efficacy of control of brown spot of pear. Sanitation methods were evaluated in nine trials in Girona (Spain) and Ferrara (Italy) over a 4-year period. The sanitation methods were leaf litter removal in December to February, and application of biological control agents (commercial formulates of *Trichoderma* spp.) to the orchard ground cover from February to May. Fungicides were also applied to the trees during the pear-growing season, scheduled according to the BSPcast model. The different methods were tested as stand-alone applications or in combination. All methods consistently reduced the disease incidence at harvest on fruit with an efficacy between 30 to 60% for leaf litter removal and more than 60% for the combination of leaf litter removal and biological control. Efficacy of sanitation alone (leaf litter removal and biological control) in reducing the brown spot level on fruit was similar in most of the trials to the efficacy obtained when fungicides were applied alone. However, integration of sanitation methods and fungicides did not improve the efficacy of disease control over the level provided by fungicides alone.

Keywords Disease management · *Pleospora allii* · *Stemphylium vesicarium*

Abbreviations
LLR Leaf litter removal
BCA Biological control agent
FUN Fungicides

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Introduction

Brown spot of pear (*Pyrus communis* L.) is a disease caused by the fungus *Stemphylium vesicarium* (Wall.) E. Simmons which is important in several pear-growing areas of Spain, Italy, France, Portugal, The Netherlands, and Belgium (Blancard et al. 1989;

Llorente and Montesinos 2002; Rossi et al. 2005a). The typical symptoms of this disease are necrotic areas on fruits, leaves and shoots. Losses may be economically important because infected fruits are unmarketable or drop prematurely from trees before harvest. On average, the losses are typically between 1 and 10% of the total production in affected areas despite the application of control measures (Llorente and Montesinos 2006). At present, disease control consists of fungicide applications after petal fall either according to a fixed schedule, every 7 or 15 days depending on the fungicide used or using a guided spray schedule provided by the BSPcast forecasting system (Llorente et al. 2000; Montesinos et al. 1995). The efficacy of control achieved by using BSPcast is similar to that of the fixed spray schedule, but BSPcast provides an average of 30% savings in the number of fungicide sprays (Llorente et al. 2000). However, efficacy of the fungicides, either for fixed or BSPcast guided strategies, is limited when the disease pressure is high due to inoculum presence, favourable environmental conditions, or susceptible pear cultivars.

The pathogen overwinters as pseudothecia of *Pleospora allii* (Rabenh.) Ces.&De Not in dead plant material either from pear trees or from herbaceous plants of the meadow (Llorente and Montesinos 2006; Rossi et al. 2005c). Maturation of the pseudothecia in dead pear leaves needs high relative humidity and temperatures between 5 and 25°C with the optimum at 10 to 15°C (Llorente and Montesinos 2004). After maturation, ascospores are released and can produce infections on pear (Llorente et al. 2006; Rossi et al. 2005b). Despite the recent advances in the knowledge of the disease, the role of this inoculum in the disease cycle is not completely known because ascospores are mainly released from March to May, too early in relation to the start of epidemics (Llorente and Montesinos 2006). It has been hypothesized that ascospores trigger the saprophytic colonization of the plant material on the orchard floor and later, when the environmental conditions are favorable, the resulting mycelium produces conidia which become airborne and infect pear trees during the vegetative period (Llorente and Montesinos 2006; Rossi et al. 2005c).

As in other fungal diseases, sanitation methods can decrease the inoculum pressure by disrupting the disease cycle at the overwintering sexual phase. For example, in apple orchards leaf litter removal (leaf shredding) and fungal antagonist (*Athelia bombacina* and *Microsphaeropsis ochracea*) application significantly decreased of ascosporic inoculum to control apple scab (Gomez et al. 2007; Holb 2006 and 2008; Holb et al. 2006; Sutton et al. 2000; Vincent et al. 2004). We have previously reported that in microplots in orchards affected by brown spot of pear, shredding or removing leaves from the orchard floor were highly effective in reducing the number of *P. allii* ascospores to undetectable levels, and biological control methods consisting of *Trichoderma* spp. formulations were only partially effective (Llorente et al. 2006). Nevertheless, *Trichoderma*-based products were capable of colonizing dead leaves of pear and a grass plant (*Digitaria sanguinalis*), and reducing the production of *S. vesicarium* conidia (Rossi and Pattori 2009). However, the effect of sanitation methods applied to the orchard floor in autumn-winter was not studied due to the small plot size used in previous studies (Llorente et al. 2006). In spite of a reduction of the inoculum, decreased inoculum levels may still develop disease under favourable conditions (Campbell and Madden 1990). For this reason, mesoscale field trials in affected pear orchards are necessary to evaluate the effect of sanitation measures on disease control.

The objective of the present study was to evaluate at the mesoscale (orchard) level the effect on brown spot disease of sanitation methods consisting of combinations of leaf litter removal and biological control agents. The experiments were performed in naturally-infected pear orchards to determine if disease control achieved by the standard schedule using fungicides sprays during the growing stage were improved by the use of additional sanitation measures.

Materials and methods

Orchard trials

Nine field trials were conducted in five pear orchards naturally infected by brown spot and located in Catalunya (Spain) and Emilia-Romagna (Italy). The experiments were performed during 2004, 2005, 2006, and 2007. For identification throughout this report, number codes are assigned to each trial

(Table 1). The pear cultivars used were 'Conference', 'Abate Fétel', and 'Passe Crassane', which are all highly susceptible to the disease. In trials 4, 5, 6, 7, 8, and 9 the level of brown spot during the previous year was high, whereas in the other trials (1, 2 and 3) the disease pressure was low.

Two sanitation methods, leaf litter removal (LLR) and application of biocontrol agents (BCA) were tested. LLR consisted of completely removing pear leaves from the ground during winter from the end of December to middle of February depending on trial (Table 2). In all cases the stage of *P. allii* pseudothecia maturation was determined to ensure that no ascospores had already been released according to a method previously described (Llorente and Montesinos 2004). Fallen pear leaves were collected using brooms either manually or with a specific device connected to a tractor, and were removed from the orchard. BCA consisted of several applications onto the ground surface of commercial formulations of *Trichoderma* spp. Two commercial products were used depending on trial: 1) Trichomic (Trichodex-AMC Chemical, Sevilla, Spain) coded as Tricho-1, consisting of a liquid formulation composed of a mixture of strains of *T. harzianum* and *T. viride* (1×10^6 cfu/ml) and commercialized as a plant growth promotion product; 2) Tusal (Newbiotecnic, Sevilla, Spain) coded as Tricho-2 composed of a mixture of *T. harzianum* and *T. viride* strains ($5x10^8$ cfu/g) presented in a powdered formulation and commercialized as a biological fungicide and plant growth promoter. Tricho-1 was applied in trials 3, 4, 5, 8 and 9, Tricho-2 in trials 6 and 7. The doses of the different *Trichoderma*-based products, expressed as amount of the commercial product, were: for Tricho-1, 4 l/ha and for Tricho-2, 1 kg/ha for the first application and 0.5 kg/ha for the remaining ones. The volume of application was 400 to 500 l/ha of the final diluted product. On each plot (500 to 600 m^2) the final volume applied was 20 to 30 litres, and the applications were made with an engine-operated portable sprayer (Table 2). The first application of BCA was made when the mean daily temperature was higher than 10°C to ensure their viability according to the instructions of the manufacturer.

Disease control during the growing season was based on fungicide applications (FUN). Fungicides were applied according to the 3-day cumulative daily infection risk (CR) provided by the BSPcast system (Llorente et al. 2000; Montesinos et al. 1995). An action threshold (i.e., CR=0.4) was used to schedule fungicide sprays: fungicides were applied after petal fall whenever the action threshold was reached, until two weeks before harvest (i.e., in August for 'Conference' and 'Abate Fétel', and October for 'Passe Crassane'). The type of fungicides used were dependent on trial (Table 2), and were: captan (150 g a.i./hl; Merpan 80, Aragonesas-AgroSA, Madrid, Spain; Captanbayer, BAYER, Filago, Italy), copper hydroxide (150 g a.i./hl; Hidrocobre 50 Alintra, IQVSA, Barcelona, Spain), copper oxychloride (50 g a.i./hl; Cobreluq 50, LUQSA, Lleida, Spain; Cuprossil, ISAGRO, Milano, Italy), kresoxim-methyl (10–14 g a.i./hl; Stroby, BASF, Ludwishafen, Germany) and thiram (200 g a.i./hl; Thiram 80, Aragonesas-AgroSA, Madrid, Spain) and the final volume applied was between 800 to 1,000 l/ha. Fungicides were applied with an engine-operated portable sprayer (Stihl model SR400, Waiblingen, Germany) or a 2,000-litre commercial sprayer (Hardi model Mercury, Taastrup, Denmark; Makato model Ecopower, Lleida, Spain).

Table 1 Characteristics of trials performed for evaluating the effect of different treatments aimed at controlling brown spot of pear by sanitation

Trial	Year	Country	Orchard location	Pear cultivar	Treatment plot size (m^2)
1	2004	Spain	St Pere (Girona)	Conference	500
2	2004	Spain	St Iscle (Girona)	Abate Fétel	500
3	2005	Spain	St Pere (Girona)	Conference	500
4	2005	Spain	Fornells (Girona)	Passe Crassane	500
5	2006	Spain	Fornells (Girona)	Passe Crassane	500
6	2007	Spain	Viladamat (Girona)	Conference	500
7	2007	Spain	Fornells (Girona)	Passa Crassane	500
8	2005	Italy	Vigarano (Ferrara)	Abate Fétel	600
9	2006	Italy	Vigarano (Ferrara)	Abate Fétel	600

Table 2 Treatments performed in orchard trials for evaluating the effect of different sanitation methods aimed at controlling brown spot of pear

Trial	Code[a]	Sanitation methods		Fungicide application period of sprays—fungicides used[b]
		Leaf litter removal date	Biological control dates of application—product	
1	FUN	–[c]	–	13 March to 16 August—Cp (1), KM(1), Th (11)
	LLR+FUN	3 January	–	13 March to 16 August—Cp (1), KM(1,) Th (11)
2	FUN	–	–	10 March to 1 September—CO(3), Th (14)
	LLR+FUN	15 January	–	10 March to 1 September—CO(3), Th (14)
3	FUN	–	–	15 March to 22 August—Km (2), Th (12)
	LLR+BCA+FUN	11 January	14 March, 30 March—Tricho-1	15 March to 22 August—Km (2), Th (12)
4	NT	–	–	–
	FUN	–	–	04 May to 27 September—Th (12), Km (2)
	LLR	23 December	–	–
	LLR+FUN	23 December	–	04 May to 27 September—Th (12), Km (2)
	LLR+BCA	23 December	10 February, 14 March—Tricho-1	
	LLR+BCA+FUN	23 December	10 February, 14 March—Tricho-1	04 May to 27 September—Th (12), Km (2)
5	NT	–	–	–
	FUN	–	–	12 May to 15 September—Th (6), Km (2)
	LLR	3 February	–	–
	LLR+FUN	3 February	–	12 May to 15 September—Th (6), Km (2)
	LLR+BCA	3 February	24 February, 13 March, 3 April—Tricho-1	
	LLR+BCA+FUN	3 February	24 February, 13 March, 3 April—Tricho-1	12 May to 15 September—Th (6), Km (2)
6	LLR+FUN	18 January	–	10 March to 14 August—Co(2), Ch (6)
	LLR+BCA+FUN	18 January	13 March, 26 April—Tricho-2	10 March to 14 August—Co (2), Ch (6)
7	NT	–	–	–
	FUN	–	–	02 May to 20 August—Th (9)
	BCA		22 February, 13 March, 16 April, 16 May—Tricho-2	–
	LLR+BCA	14 February	22 February, 13 March, 16 April, 16 May—Tricho-2	–
	LLR+BCA+FUN	14 February	22 February, 13 March, 16 April, 16 May—Tricho-2	02 May to 20 August—Th (9)
8	NT	–	–	–
	FUN	–	–	16 June to 31 August—Th+Co+Km (3), Cp (3), Km (1)
	LLR+FUN	15 December	–	16 June to 31 August—Th+Co+Km (3), Cp (3), Km (1)
	LLR+BCA+FUN	15 December	30 March, 18 April—Tricho-1	16 June to 31 August—Th+Co+Km (3), Cp(3), Km (1)
9	NT	–	–	–
	FUN	–	–	08 May to 13 August—Th (1), Th+Cp (1), Km+Cp (4), Cp (1)
	LLR+FUN	20 December	–	08 May to 13 August—Th (1), Th+Cp (1), Km+Cp (4), Cp (1)

LLR+BCA	20 December	21 April, 08 May—Tricho-1	–	
LLR+BCA+FUN	20 December	21 April; 08 May—Tricho-1	08 May to 13 August—Th (1), Th+Cp (1), Km+Cp (4), Cp (1)	

[a] *BCA* Biological control using commercial formulates of *Trichoderma* spp. where Tricho-1 corresponds to Trichomic and Tricho-2 to Tusal.; *FUN* Fungicide applications during the growing season; *LLR* Leaf litter removal; *NT* Non-treated control

[b] Fungicides applied were: *Cp* captan; *Ch* copper hydroxide; *Co* copper oxychloride; *Km* Kresoxim-methyl; *Th* thiram. The number total of applications are indicated in *parentheses*

[c] Treatment not done

Depending on the trial the treatments were applied alone (BCA, LLR) or in different combinations (BCA+LLR, LLR+FUN or BCA+LLR+FUN), as well as a non-treated control (NT) (Table 2). Within each trial, an additional control consisted of the application of fungicides alone that was compared to sanitation methods. This was because the objective of the work was to evaluate if the efficacy of disease control achieved by the standard schedule using fungicide applications during the growing stage was increased using sanitation methods on the soil.

Each treatment was arranged in a single plot of 500–600 m^2 that was selected at random within each orchard. The dominant wind direction was considered at the time of distributing treatment plots within each orchard, to avoid the interference of the sources of inoculum between the plots where the leaves of the ground were removed and those where the leaves remained. In each plot fifteen (trials 1, 2, 3, 4, 5, 6 and 7), twenty (trial 8) or six (trial 9) single-trees were randomly selected for disease assessment, thus considering each single-tree as pseudoreplication. In the present study the experimental design was based on pseudoreplicates because in mesoscale studies in commercial orchards true repetitions of the treatments are not easy to be performed due to several restrictions (large size of the replicates, high cost of crop losses, logistic problems of treatment applications, sampling, etc.). Pseudoreplication is frequently used in ecological studies, when comparing multiple samples from the same experimental unit between different environmental systems or treatments (Garrett et al. 2004; Hurlbert 1984). According to Hurlbert (Hurlbert 1984) the experimental design used herein was considered as a clumped segregation design.

Weather conditions during trials

Environmental parameters were monitored with CR10X dataloggers (Campbell Scientific Ltd., Leicester, UK) connected to temperature-relative humidity (model HMP35AC), wetness (model 236) and rainfall (model ARG100) sensors. Automatic weather stations were placed into the experimental plots or in neighbour orchards. Temperature and relative humidity were measured every 10 min and wetness and rainfall every 20 s. Mean temperature and relative humidity, duration of wetness, and total rainfall were recorded every hour.

Disease assessment

On fruit, disease incidence (% of affected fruits) and severity (number of lesions per fruit) were assessed considering all fruit present in the band within 0.5 m and 2 m above the soil in fifteen (trials 1, 2, 3, 4, 5, 6 and 7), twenty (trial 8) or six (trial 9) single-trees per plot. Assessments were performed every 15 to 20 days from fruit set to harvest. At harvest all fruit were assessed on the previously described trees.

Disease severity on leaves was assessed on 10 leaves of four shoots per tree located on both sides of the row in fifteen (trials 1, 2, 3, 4, 5, 6 and 7), twenty (trial 8) or six (trial 9) single-trees per plot. Each leaf was assigned to a severity class based on the approximate number of lesions, as follows: class 0 (no lesions), class 1 (1 to 5 lesions), class 2 (6 to 25 lesions), and class 3 (more than 25 lesions). Mean disease severity of each plot was calculated using the following formula:

$$S = \sum_{n=1}^{N} I_n / 3 \cdot N$$

where: S is the index of relative disease severity (from 0 to 1); I_n is the disease severity class of each n^{th} leaf; N is the total number of leaves assessed; and 3 is the maximum level of severity. Disease incidence was calculated as the percentage of leaves with at least one lesion.

Data analysis

Data of disease progress on fruit and leaves were analyzed using the area under disease progress curve (AUDPC)(Campbell and Madden 1990) on those trials where the disease progression was assessed. Values were standardized by dividing AUDPC data by the duration of epidemics in days. For each trial, AUDPC was analyzed for disease incidence and severity separately.Data of disease incidence and severity on fruits and leaves at harvest were also analyzed.

Because we used a pseudoreplication experimental design, statistical treatment of the data required non-conventional tests like the Linear Mixed Models analysis (Garrett et al. 2004; Schabenberger and Pierce 2002). Data were analyzed using Proc MIXED with the *ddfm= satterth* option in the model statement (SAS system v.8.02, SAS Institute Inc. North Carolina, USA). Least square means of treatments were compared using the *pdiff* option in the LSMEANS statement of the Proc MIXED and least significant difference (LSD) values were calculated using the standard errors and t values from the pairwise comparison (P=0.05).

Finally, the efficacy of control measures was determined in the trials where a non-treated control was included; in trials 1, 2, 3, and 6, non-treated controls were not included due to agronomic constraints. Efficacy was calculated for mean disease incidence and severity on fruits and leaves at harvest, and for mean AUDPC-incidence and AUDPC-severity observed on fruits and leaves in trials where disease progression was determined. Efficacy of treatments was calculated using the following formula:

$$E = [1 - (y_t / y_{nt})] \times 100$$

where: E is the efficacy of the method; y_{nt} the disease level in the non-treated control (incidence or severity at harvest or AUDPC-incidence and AUPDC-severity); and y_t the disease level in treated fruit or leaves.

Results

Weather conditions

Dynamics of temperature, wetness duration and rainfall from January to August are presented for trials 1, 3, 4, 5, 6 and 7 (Fig. 1). In most of the trials the rainfall periods were between late January to May and during August. In the majority of trials, except in trials 5 and 7, the leaves were removed on December–January before the rain period started. Trial 5 showed a long dry period from April to July, and trials 6 and 7 showed a dry period from June to July. The wetness varied along the year for the different trials and the longer daily wetness periods were due to rain but also to dew. Mean temperature values were consistently higher than 10°C at the end of February to March and then from the middle of April increased considerably when the BCA was applied.

Effects of treatments on disease progress on fruit

Disease progression on fruit was assessed in trials 1, 4, 5, 7, 8, and 9. In trial 2 and 6, the disease

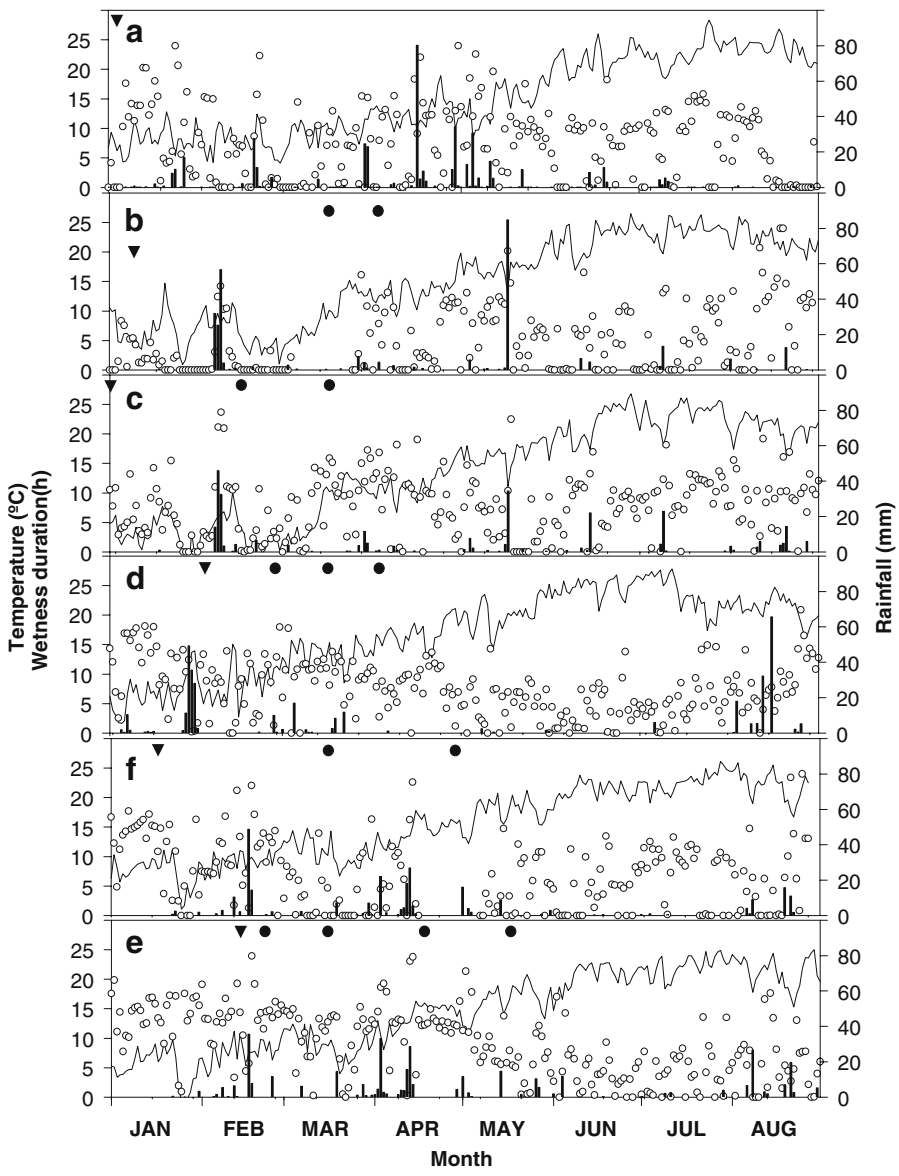

Fig. 1 Dynamics of mean temperature, daily wetness duration and rainfall in trials 1 (**a**), 3 (**b**), 4 (**c**), 5 (**d**), 6 (**e**) and 7 (**f**). Dates of sanitation treatments are indicated and corresponded to leaf litter removing (▼) or biological control (●)

on fruit was not evaluated because the number of fruit was very low due to either low fruit set (trial 2) or low number of flowers in young trees (trial 6). In trials 1 and 3, the level of disease was low and few fruit showed lesions at harvest despite the fact that the climatic conditions were favourable for infection.

The percentage of fruit affected was higher in non-treated plots (13 to 80% depending on trial) compared to treated plots (Fig. 2). The disease severity progress curves for fruit were very similar to disease incidence progress curves (data not shown).

In trial 1, treatments showed no effects on AUDPC-incidence or AUDPC-severity. In trials 4, 5, 7, 8, and 9, treatments influenced AUDPC-incidence (Table 3). LLR alone decreased the disease in two trials where it was applied (trials 4 and 5). The combination of LLR and BCA, with no fungicide sprays, decreased the disease in all the trials where it was tested (trials 4, 5, 7, and 9). When fungicides were applied during the vegetative period, the level of disease control was high whatever the method of removal of the overwintering inoculum used, but in none of these trials was there complete control. In

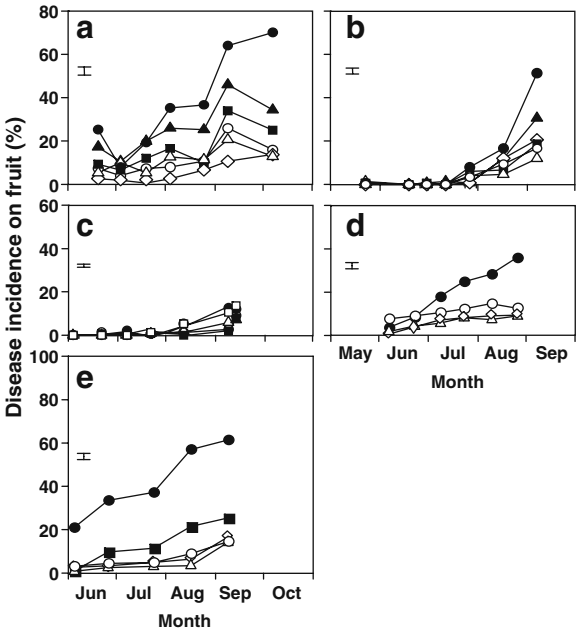

Fig. 2 Progress of the incidence of pear fruit affected by brown spot in trials 4 (**a**), 5 (**b**), 7 (**c**), 8 (**d**) and 9 (**e**). Non-treated control (●); fungicide applications, FUN (○); leaf litter removing, LLR (▲); biological control using Tricho-1 in trials 4, 5, 8, and 9 and Tricho-2 in trial 7, BCA (□); combination of LLR and FUN (△); combination of LLR and BCA (◊); and combination of LLR, BCA, and FUN (■). Bars correspond to the mean standard error of all treatments

trials 4 and 9 the combination of LLR, BCA and FUN decreased the AUDPC-incidence, not only compared to the non-treated control but also to the fungicide treatment alone. Efficacy of the applications of Tricho-2 applied alone (trial 7) did not result in disease control.

The AUDPC-severity on fruit was analyzed in trials 1, 4, 5, and 7. As previously mentioned, no differences between the treatments were observed in trial 1. In trials 4, 5, and 7 the results were very similar to those obtained using AUDPC-incidence as described above (Table 3).

Effects of treatments on disease progress on leaves

The progression of disease incidence and severity on leaves was evaluated in trials 1, 4, 5, 6, 7, 8 and 9 (Fig. 3). In most cases there was more disease on leaves than on fruit, and the efficacy of disease control by the various treatments was lower on leaves than on fruit. The AUDPC-incidence differed among treatments in all trials, while the AUDPC-severity showed differences in trials 1, 4, 5, 6 and 7 but not in trial 8 (Table 3).

The AUDPC-incidence on leaves treated only with fungicides (FUN) decreased compared to the non-treated controls in 4 out of 5 trials (Table 3). In all trials where tested, the treatments with both LLR and FUN decreased the AUDPC-incidence compared to NT, but not compared to FUN alone (trials 4, 5, 8 and 9). When LLR was applied alone or in combination with BCA no differences with the non-treated control were observed in 3 out of 4 trials containing these treatments (trials 4, 5, 7 and 9). Finally, the combination of LLR, BCA and FUN in any of the 6 trials increased disease control compared to FUN treatment.

Disease on fruit at harvest

In trials 4, 5, 7, 8, and 9, all treatments reduced disease incidence and severity on fruit at harvest in relation to the non-treated control, except BCA and LLR+BCA+FUN in trial 7 (Table 4). Treatments consisting of combination of LLR and BCA (trials 4, 5, 7, and 9) showed that disease incidence was significantly lower than in non-treated controls. In all cases where fungicides were applied, disease control efficacy was high regardless of the method of sanitation applied.

Disease on leaves at harvest

Disease incidence on leaves in non-treated controls ranged from 19.7% to 99.0%. Disease severity was determined in all trials, except in trial 9 (Table 4). Differences in disease incidence were observed in trials 1, 4, 5, 6, 7, 8, and 9 (7 out of 9 trials). LLR alone did not result in disease control (trials 4 and 5). However, when fungicides were applied during the growing season in combination with LLR in winter (trials 4, 5, 8 and 9), disease incidence decreased in relation to the non-treated control in all trials. In most trials in which FUN was applied alone or in combination with the sanitation methods, the efficacy in disease control was high and similar between treatments (trials 2, 3, 5, 7, and 9). However, in trials 4, 6, 7 and 8, the combined treatment of LLR, BCA, and FUN increased the level of control of disease incidence compared to FUN or FUN+LLR. When disease severity was analyzed, differences were

Table 3 Effects of different treatments aimed at controlling brown spot of pear by sanitation on progress of disease incidence and severity on fruit and leaves

		Fruit		Leaves	
Trial	Treatments[a]	AUDPC[b]-incidence	AUDPC-severity	AUDPC-incidence	AUDPC-severity
1	FUN	4.1	0.041	33.5 a	0.13 a
	LLR+FUN	2.5	0.026	24.1 b	0.10 b
	P-value	n.s.[c]	n.s.	***	***
4	NT	39.5 a[d]	0.976 a	74.24 a	0.36 ab
	FUN	12.5 c	0.318 b	63.32 cd	0.27 c
	LLR	27.6 b	0.944 a	77.96 a	0.38 a
	LLR+FUN	12.2 c	0.353 b	66.08 bc	0.27 c
	LLR+BCA	17.8 c	0.334 b	69.71 ab	0.34 b
	LLR+BCA+FUN	6.1 d	0.111 b	51.28 d	0.20 d
	P-value	***	***	***	***
5	NT	18.1 a	0.235 a	39.32 a	0.17 a
	FUN	7.6 b	0.094 b	33.39 bc	0.14 bc
	LLR	10.5 b	0.089 b	39.61 a	0.17 ab
	LLR+FUN	8.4 b	0.111 b	31.25 c	0.13 c
	LLR+BCA	7.8 b	0.034 b	38.59 ab	0.16 abc
	LLR+BCA+FUN	5.2 b	0.054 b	41.61 a	0.19 a
	P-value	***	***	***	***
6	LLR+FUN	–[e]	–	14.4 a	0.12 a
	LLR+BCA+FUN	–	–	7.4 b	0.04 b
	P-value			***	***
7	NT	5.3 a	0.054 a	38.7 a	0.16 a
	FUN	1.3 c	0.013 b	34.5 ab	0.15 ab
	BCA	4.8 ab	0.057 a	29.9 b	0.12 bc
	LLR+BCA	0.6 c	0.005 b	33.3 b	0.13 bc
	LLR+BCA+FUN	2.3 bc	0.026 ab	29.2 b	0.12 c
	P-value	***	**	***	***
8	NT	19.7 a	–	12.4 a	0.10
	FUN	6.2 b	–	7.9 b	0.08
	LLR+FUN	11.4 b	–	6.9 b	0.09
	LLR+BCA+FUN	6.6 b	–	9.3 b	0.06
	P-value	***		**	n.s.
9	NT	41.9 a	–	47.45 a	–
	FUN	6.7 c	–	24.54 b	–
	LLR+FUN	6.1 cd	–	23.87 b	–
	LLR+BCA	13.8 b	–	47.10 a	–
	LLR+BCA+FUN	4.2 d	–	24.85 b	–
	P-value	***		***	

[a] *BCA* Biological control agent using Tricho-1 in trials 1, 4, 5, 8 and Tricho-2 in trials 6, 7; *LLR* Leaf litter removal; *FUN* Fungicide applications during the growing season; *NT* Non-treated control

[b] *AUDPC* area under disease progress curve. Values of AUDPC were standardized by dividing calculated values by duration of epidemic in each trial

[c] Level of significance ***: $P<0.01$; **:$0.01<P\leq0.05$; *n.s.*: not significant

[d] Least significant differences were calculated using the pairwise comparisons of LSMEANS. Values followed by the same letter are not different at $P=0.05$

[e] Treatment not evaluated

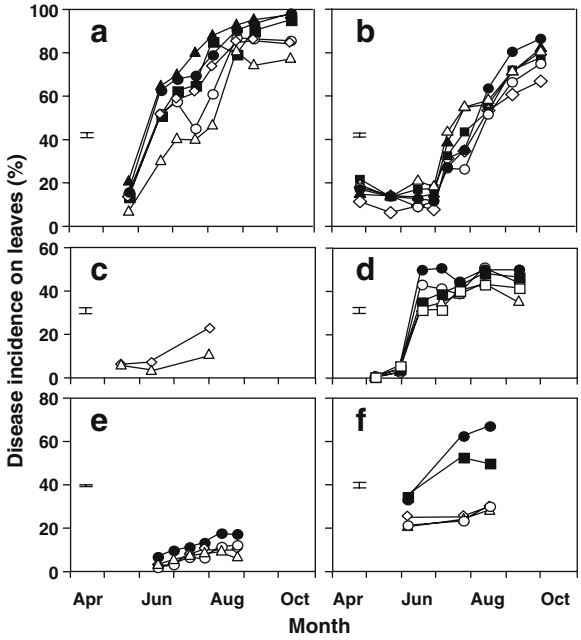

Fig. 3 Progress of the incidence of pear leaves affected by brown spot in trials 4 (**a**), 5 (**b**), 6 (**c**), 7 (**d**), 8 (**e**) and 9 (**f**). Non-treated control (●); fungicide applications, FUN (○); leaf litter removal, LLR (▲); biological control using Tricho-1 in trials 4, 5, and 9 and Tricho-2 in trials 7 and 8, BCA (□); combination of LLR and FUN (△); combination of LLR and BCA (◊); and combination of LLR, BCA, and FUN (■). Bars correspond to the mean standard error of all treatments

significant in 4 trials out of 9 (trials 4, 5, 6, and 7) indicating that all treatments where fungicide was applied alone or in combination with LLR had a similar efficacy in controlling the disease.

An overview of the efficacy of disease control

A reduction of disease incidence and severity on both fruit and leaves at harvest, as well as during the whole epidemic (measured as AUDPC) was observed in the trials where a non-treated control was included (trials 4, 5, 7, 8 and 9) (Table 5).

The efficacy of control was higher on fruit than on leaves since most of trials showed an efficacy higher than 60% on fruit and lower than 30% on leaves. When the efficacy of control was analyzed on fruit all strategies were effective in decreasing disease incidence and AUDPC. Treatments with FUN applied alone or combined with sanitation methods (LLR+FUN or LLR+BCA+FUN) gave the highest efficacy (>60%). Sanitation methods applied alone (LLR) reduced the disease in comparison to non-treated control about 30 to 60%. When sanitation methods were applied in combination (LLR+BCA) the efficacy was higher than 60% in most trials and similar to the fungicide treatment when applied alone (FUN) or in combination with sanitation methods (FUN, LLR+FUN or LLR+BCA+FUN).

Discussion

Spraying fungicides to trees is the main strategy to manage brown spot of pear in the areas of the world where the disease is of economic importance. Unfortunately, the control achieved by repeated fungicide applications is not satisfactory under favourable environmental conditions for brown spot, highly susceptible cultivars, and high disease pressure (Llorente and Montesinos 2006). Thus, additional methods are needed to increase the efficacy of disease control, as for example sanitation measures to reduce inoculum.

In apple scab, there is a direct relationship between the concentration of airborne ascospores and disease severity (Aylor and Kiyomoto 1993; Carisse et al. 2000; Sutton et al. 2000). Removing leaf litter during the winter is a control method used for several plant diseases including apple scab because this material is the source of ascospores (Gomez et al. 2007; Holb 2006 and 2008; Holb et al. 2006; Sutton et al. 2000; Vincent et al. 2004). Sutton et al. (2000) showed that when apple leaf litter was completely shredded during fall or early spring, the risk of scab was reduced by 80 to 90%. However, when 10 to 35% of the leaf litter remained unremoved, the risk of scab was only reduced by 50 to 65%.

The experiments performed in the present work were done in different climatic areas, during several years, and with three pear cultivars. In spite of the wide range of conditions, the results obtained upon application of sanitation methods were consistent. Although the disease reduction upon decreasing the level of the overwintering inoculum was lower than that expected according to the previous research based on ascospore release (Llorente et al. 2006), it was significant. Both final disease incidence and severity were reduced on fruit when the leaf litter was removed in comparison to the non-treated control, with no fungicide application. Also, almost

Table 4 Effects of different treatments aimed at controlling brown spot of pear by sanitation on disease incidence and severity on fruit and leaves at harvest

Trial	Treatments[a]	Fruit		Leaves	
		Incidence[b]	Severity [c]	Incidence	Severity
1	FUN	1.8	0.02	68.0 a	0.29
	LLR+FUN	1.3	0.02	56.9 b	0.25
	P-value	n.s.[d]	n.s.	***	n.s.
2	FUN	–[e]	–	58.3	0.77
	LLR+FUN	–	–	50.7	0.71
	P-value	–	–	n.s.	n.s.
	FUN	5.9	0.06	31.7	0.07
3	LLR+BCA +FUN	4.2	0.04	19.7	0.12
	P-value	n.s.	n.s.	n.s.	n.s.
4	NT	70.1 a[f]	1.87 a	99.0 a	0.60 a
	FUN	15.9 cd	0.20 d	85.7 b	0.37 b
	LLR	34.4 b	0.55 b	97.7 a	0.61 a
	LLR+FUN	12.9 d	0.16 d	84.3 b	0.36 b
	LLR+BCA	25.0 bc	0.33 c	95.3 a	0.53 a
	LLR+BCA +FUN	13.8 d	0.17 d	77.3 c	0.29 b
	P-value	***	***	***	***
5	NT	51.3 a	0.71 a	86.3 a	0.40 a
	FUN	16.7 c	0.21 bc	74.8 ab	0.33 bc
	LLR	30.7 b	0.34 b	81.8 a	0.39 ab
	LLR+FUN	20.7 bc	0.25 bc	66.8 b	0.30 bc
	LLR+BCA	18.9 bc	0.21 bc	77.7 ab	0.35 abc
	LLR+BCA +FUN	12.0 c	0.34 c	80.3 a	0.38 ab
	P-value	***	***	***	**
6	LLR+FUN	–	–	29.7 a	0.18a
	LLR+BCA +FUN	–	–	14.0 b	0.06b
	P-value			***	***
7	NT	12.7 a	0.15 ab	50.2 a	0.21 a
	FUN	2.7 b	0.03 b	44.5 ab	0.17 ab
	BCA	10.7 a	0.36 a	41.8 bc	0.17 ab
	LLR+BCA	2.0 b	0.01 b	46.8 ab	0.19 a
	LLR+BCA +FUN	6.0 ab	0.07 b	35.3 c	0.13 b
	P-value	***	**	***	***
8	NT	42.7 a	0.22 a	17.2 a	0.05
	FUN	12.1 b	0.06 b	13.8 b	0.03
	LLR+FUN	7.2 bc	0.03 b	9.9 c	0.04
	LLR+BCA +FUN	6.4 c	0.03 b	6.2 d	0.02
	P-value	***	***	***	n.s.
9	NT	61.4 a	0.34 a	66.8 a	–
	FUN	14.6 c	0.06 b	29.8 c	–
	LLR+FUN	16.9 c	0.06 b	29.6 c	–
	LLR+BCA	25.4 b	0.09 b	50.0 b	–
	LLR+BCA +FUN	14.3 c	0.05 b	27.9 c	–
	P-value	***	***	***	

[a] *BCA* Biological control agent using Tricho-1 in trials 1, 3, 4, 5, 8, 9 and Tricho-2 in trials 6, 7; *LLR* Leaf litter removal; *FUN* Fungicide applications during the growing season; *NT* Non-treated control

[b] Disease incidence as a percentage of affected fruit or leaves

[c] Disease severity as the number of lesions on fruit and relative index on leaves (the index varies from 0 to 1)

[d] Level of significance ***: $P<0.01$; **:$0.01<P\leq0.05$;*n.s.*:not significant

[e] Treatment not evaluated

[f] Least significant differences were calculated using the pair-wise comparison of LSMEANS. Values followed by the same letter are not different at $P=0.05$

complete removal of pear leaf litter during winter decreased the incidence and AUDPC of brown spot on fruit only partially. Incidence at harvest and AUDPC were reduced from 30 to 60% of affected fruit. The efficacy of this treatment was improved by combination with BCA or FUN. Similarly the efficacy of sanitation methods in controlling apple scab is greatly dependent on location and climatic conditions (Holb et al. 2006), or cultivar type (Holb 2008). Contrarily, disease levels in leaf spot disease of alfalfa caused by *Stemphylium botryosum*, were almost independent of the initial level of infected crop debris as a source of inoculum, and disease development was almost the same in plots where infected debris had been removed or not (Duthie and Campbell 1991). One explanation for the lack of complete disease control is incomplete removal of the overwintering inoculum. In brown spot of pear, the ascospores produced by the overwintered pseudothecia of *P. allii* seem to colonize the dead plant material of the orchard floor saprophytically rather than producing infections on pear tissue (Llorente and Montesinos 2006; Rossi et al. 2005c; Rossi et al. 2008). Later, under warm and humid conditions, the asexual phase of the fungus may produce conidia on the leaf litter and dead grasses, which then

Table 5 A global analysis of the efficacy of different treatments aimed at controlling brown spot of pear by sanitation expressed as reduction of the disease incidence at harvest or of area under disease progress curve (AUDPC-incidence) compared to a non-treated control on fruit and leaves

Treatment[a]	Number of trials performed	Incidence at harvest			AUDPC-incidence		
		Efficacy range (%)			Efficacy range (%)		
		<30[b]	30–60	>60	<30	30–60	>60
Fruit							
FUN	5	–	–	5	–	1	4
LLR	2	–	2	–	–	2	–
LLR+FUN	4	–	1	3	–	2	2
LLR+BCA	4	–	1	3	–	2	2
LLR+BCA+FUN	5	–	1	4	–	1	4
Leaves							
FUN	5	4	1	–	3	2	–
LLR	2	2	–	–	2	–	–
LLR+FUN	4	2	2	–	2	2	–
LLR+BCA	4	4	–	–	4	–	–
LLR+BCA+FUN	5	3	1	1	3	2	–

[a] *BCA* Biological control using Tricho-1 in trials 4, 5, 8, 9 and Tricho-2 in trial 7; *LLR* Pear leaf litter removal; *FUN* Fungicide applications during the growing season

[b] Number of trials where the efficacy of control was >30%, between 30–60% and >60% in comparison to non-treated control

become airborne and infect pear trees (Llorente and Montesinos 2006; Rossi et al. 2005a; Rossi et al. 2005c). The role of this process as a source of conidia during the pear growing season is reinforced by the fact that brown spot lesions on pear leaves and fruits generally do not sporulate, contrary to what happens for apple scab. Therefore, small amounts of overwintering inoculum remaining after sanitation may be sufficient to increase the inoculum potential by saprophytic growth. Additionally, it is not excluded completely that some lesions were caused by the incoming ascospores or conidia from neighboring plots or orchards, but the size of the plots used herein was big enough to minimize the interaction between plots. Also, it has been described that spores of *P. allii* and *S. vesicarium* can not be transported over long distances (*unpublished data*). Thus, the contribution of lesions arising from incoming inoculum to the total amount of disease probably was low.

Interestingly, for apple scab (Gadoury and MacHardy 1986),the reduction of the potential ascospore dose delayed the disease progression, decreasing the disease level only at the beginning of the epidemic, but not necessarily reduced the final amount of the disease at harvest in the absence of additional control measures. In the case of disease progress curves presented here, the disease level was reduced during the whole epidemic when the leaf litter was removed alone or in combination with BCA or FUN. The results obtained in trials 4 and 5 performed in the same orchard with the same treatments but in two different years were remarkable. There were slight differences between years in the AUDPC that may be explained due to the period without rain observed from June to the middle August in trial 5. As a consequence of the climatic conditions, in trial 4 the epidemics started during June on leaves and in July on fruit, whereas in trial 5 the epidemics began in July on leaves and in August on fruits. Despite this, the efficacy of the treatments was similar for both trials in relation to disease incidence on fruit at harvest.

Biological control of brown spot has been studied in the past. Strains of *Pseudomonas fluorescens* and *Pantoea agglomerans* antagonistic to *S. vesicarium* were found to be effective in preventing brown spot infections when sprayed on potted pear plants in the greenhouse (Montesinos et al. 1996). However, the same strains were ineffective when tested as spray

applications during the growing season on orchard trials (data not published). Also, tree spray applications of strains of *Trichoderma koningii*, and *T. viride* have been tested in orchards previously with no significant control of brown spot of pear (Llorente and Montesinos 2006). In these cases, control failure was attributed to either low survival of the biocontrol agent, poor colonization of pear leaves, or decreased competition with autochthonous microorganisms in the phyllosphere (Llorente and Montesinos 2006). *Trichoderma* spp treatments reduced the production of ascospores of *P. allii* in orchards (Llorente et al. 2006). *Trichoderma* spp. also reduced the production of *S. vesicarium* conidia on dead pear and *Digitaria sanguinalis* leaves by >99% six weeks after application (Rossi and Pattori 2009).

In the present work, biological control was targeted to decrease the saprophytic survival of the pathogen in soil debris on the orchard floor during late winter. It was expected that the environmental conditions (particularly, moisture, temperature, and nutrient availability) in this target site may favour survival and saprophytic growth of the biocontrol agent, compared to the stress that the microorganism may experience in the phylllosphere during the hot and dry summer months of the Mediterranean climate. Since products were applied between mid-February and April, when the *P. allii* pseudothecia have been already formed, the biocontrol mechanism used by the applied *Trichoderma* spp. was probably mycoparasitism or competitive interaction (Carisse and Rolland 2004; Faize et al. 2003; Howell 2003; Llorente et al. 2006; Philion et al. 1997). In the present study, the biological control agents were mainly used as a tool for improving efficacy of the leaf litter removal. Efficacy of the combination of leaf litter removal and subsequent application of a biological control agent was high: disease levels decreased in all the trials where this sequential treatment was tested, reinforcing the idea that leaf litter material plays an important role as a reservoir of the overwintering inoculum. In addition, in these trials, the biological treatment was applied only in early spring. Repeated applications of the biocontrol agent during the pear-growing season may increase the efficacy of the biocontrol, since conidia of *S. vesicarium* are produced on the dead plant tissue on the orchard floor season long (Rossi et al. 2005c; Rossi and Pattori 2009).

Brown spot control based only on the reduction of the overwintered inoculum in the orchard ground had the same level of efficacy in most of the trials as the application of fungicides alone to trees during the vegetative period according to BSPcast. However, sanitation did not increase the efficacy of fungicide applications when sanitation and fungicides were combined. Nevertheless, reduction of the pathogen population through LLR and BCA with *Trichoderma* spp. potentially has several benefits, especially at mid-term: 1) reducing the number of fungicide sprays during the growing season; 2) reducing the risk for fungicide-resistant populations of *S. vesicarium* (Alberoni et al. 2005); 3) increasing efficacy of brown spot control in organic pear production where disease control is based mainly on copper derivative applications which have low efficacy.

Sanitation through leaf litter removal and application of biocontrol agents can be incorporated into an integrated brown spot management program as with apple scab (MacHardy et al. 2001). Direct practical applications of collector adapters, disc cultivation and ploughing were relatively efficient against apple scab and showed a reduction of spur-leaf scab incidence (Holb 2007). Removal of fallen leaves from the orchard floor during winter can be made with commercially available leaf collector adapters for most farm tractors, and its effect can be reinforced, as well as extended to the dead leaves of the herbaceous plants of the orchard floor, by the ground application of effective *Trichoderma* formulations.

Continuing research is focused on selecting more effective biological control agents and their application during the whole year. Also, development of new fungicides with higher efficacy against brown spot of pear is needed. Furthermore, it is necessary to understand the reasons why the efficacy of control is not complete, in spite of the combination of three control methods (leaf litter removal, biological control agents, and fungicide applications) that act in different stages of the disease cycle.

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