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Biological Control 32 (2005) 305–310
Biological Control
www.elsevier.com/locate/ybcon

Biological control of postharvest brown rot (*Monilinia* spp.) of peaches by field applications of *Epicoccum nigrum*

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Received 27 July 2004; accepted 25 October 2004
Available online 2 December 2004

Abstract

Seven field experiments were carried out in peach orchards located in Spain, Italy, and France in 2001 and 2002 to develop an effective and practical method of controlling brown rot disease caused by *Monilinia* spp. by pre-harvest applications of *Epicoccum nigrum* treatments. Three trees (100 fruits), randomly selected in each orchard, were used as the sample unit and every treatment was repeated four times. Factors considered in each orchard and year to compare *E. nigrum* and/or fungicide pre-harvest application were the time of application, fresh or formulated cells, and dose. Fresh or formulated cells (10^{6-7} conidia ml^{-1}) of *E. nigrum* need to be applied twice both at bloom and preharvest to reduce postharvest brown rot. Chemical fungicides reduced disease in French and Italian trials but not in a Spanish trial. Integrated control (biological and chemical) was efficient in controlling the pathogens. *E. nigrum* application, alone (applied 4 times) or in combination with fungicides can be considered in a disease control strategy for reducing fungicide treatments and residues. A further reduction of brown rot may be possible by a better formulation of the biological product and postharvest combined treatments.

Keywords: Biocontrol; *Epicoccum nigrum*; *Monilinia laxa*; *M. fructigena*; *M. fructicola*; Postharvest; *Prunus persica*

1. Introduction

Brown rot of peach (*Prunus persica* (L.) Batch) is caused in the European Mediterranean areas by the fungi *Monilinia laxa* (Aderh et Rulh) Honey and *Monilinia fructigena* Honey in Whetzel (De Cal and Melgarejo, 1999). A third species, *Monilinia fructicola* (Wint.) Honey, causes brown rot in Australia, South Africa, North and South America and is listed as a European quarantine organism (European and Mediterranean Plant Protection Organization, 1992). Brown rot is a serious disease in these European Mediterranean areas. Direct yield losses result from infection of flowers (flower and twig blight) and from fruit rot at preharvest, harvest, and postharvest. Postharvest losses are typically more severe than preharvest losses, and routinely occur during storage and transport, in some cases even affecting fruit at the processing stage (Hong et al., 1997). When conditions are favourable for disease development, postharvest losses may be high, reaching in some

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doi:10.1016/j.biocontrol.2004.10.010

cases values of 80–90% (Hong et al., 1997, 1998). Average fruit production by European Union countries amounts to approximately 4 million metric tons. According to data provided by "Europech," for the five year period from 1998 to 2002, the main E.U. peach producing countries include Italy, (1,017,400 tons), Spain (1,039,900 tons), Greece (780,300 tons), and France (435,200 tons) (Rodríguez Navarro, 2003).

Chemical control of flower blight phase in peach growing Mediterranean areas is done by applying benzimidazole, dicarboximide, or IBS fungicides (Hong et al., 1998). The fungicides are also used at preharvest applications to prevent fruit rot. Postharvest applications of fungicides are not allowed in Italy, but used in Spain and France (Cravedi, 2000). With the development of integrated production systems the various peach growing regions, postharvest fungicide application is no longer allowed (Malavolta et al., 2003). In addition, resistance to benzimidazole and dicarboximide, fungicides is well documented in *Monilinia* spp., especially in *M. fructicola* populations (Elmer and Gaunt, 1993; Penrose, 1990; Penrose et al., 1979; Sanoamuang and Gaunt, 1995). New environmentally friendly alternative control methods are being sought based on demands of consumers and environmental protection agencies.

The potential of *Epicoccum nigrum* Link:Fr., a component of the resident mycoflora of peach twigs and flowers (Melgarejo et al., 1985), for biological control of peach twig blight caused by *M. laxa* has been demonstrated in field experimental orchards under artificial inoculation of the pathogen (Madrigal et al., 1994; Melgarejo et al., 1986; Pascual et al., 1996). *E. nigrum* has been used previously in experiments on biocontrol of other diseases (Boland and Inglis, 1988; Zhou and Reeleder, 1990). This paper describes attempts to develop an effective and practical method of controlling brown rot disease at postharvest by using *E. nigrum* alone or in combination with fungicides by peach orchard applications. Trials were carried out during two years in three different European Mediterranean peach growing countries to assess the potential of using *E. nigrum* for brown rot control under commercial peach production.

2. Materials and methods

Seven field experiments were carried out in peach commercial orchards located in Spain, Italy, and France in 2001 and 2002 (Table 1). Trees were selected at random in each orchard. Three consecutive trees were used as the sample unit and every treatment was repeated four times. Two guard trees were used to separate sample units to avoid spray drift.

Different treatments were applied in each orchard and each year to compare preharvest application of *E. nigrum* to control *Monilinia* spp. disease at postharvest (Table 2). Treatments were applied at bloom and/or at preharvest, respectively, in the following dates: 6, 21 March and 22, 29 August 2001 in SP1; 12 March, 4 April, and 26 July, 3 August 2001 in IT1; 14 March, 4 April, and 26 July, 3 August 2002 in IT2; 19 March, 10 April, and 18, 31 July 2002 in IT3; 5, 15 March, and 9, 22 August 2001 in FR1; 5, 15 March, and 14, 21 August 2001 in FR2; and 1, 15 March, and 4, 15 July 2002 in FR3.

Fresh conidia of *E. nigrum* were produced as described in Larena et al. (2004) in a solid-state fermentation system. Briefly, the fungus was grown on a mixture of peat (Gebr. BRILL substrate GmbH KG, Georgsdorf, Germany): vermiculite (Termita, Asfaltex, S.A., Barcelona, Spain): lentil meal (1:1:0.5, w/w/w). Fifty hundred grams of the substrate described above (40% w/w water content) were placed in a plastic bag (1300 cm^3) designed for solid-state fermentation (VALMIC^R, Sacherei de Pont-Audemer S.A., Pont-Audemer, France), sealed, and sterilised by autoclaving at 1.0 $kg\,cm^{-2}$ and 120 °C for 1 h on 3 consecutive days. Bags were then inoculated with a 10^5 conidia g^{-1} dry substrate conidial suspension of *E. nigrum* produced in Petri plates containing potato dextrose agar, sealed and incubated in darkness at 20–25 °C for 10 days. After incubation, water was added to the mixture of conidia + substrate at a rate of 1:4 (w/v), the resulting spore suspensions were shaken in a rotary shaker (Bunsen, S.A., model AO400, Madrid, Spain) at 200 rpm for 10 min, and filtered through glass wool. Most conidia passed through glass wool and were concentrated by centrifugation at 10,000 rpm for 10 min. The final yield was 10^8 conidia g^{-1}

Table 1
Characteristics of trials carried out in orchards located in Spain (SP), Italy (IT), and France (FR) in 2001 and 2002

Trial	Location	Year	Cultivar	Planting date	Planting distance (m)	Harvest date
SP1	Alfarrás, Lleida, Spain	2001	Caldesi 2020	February 1994	5 × 4	4/9
IT1	Salerano sul Lambro, Lombardia, Italy	2001	Venus	February 1993	6 × 5	6/8
IT2	Villafranca, Veneto, Italy	2001	Fantasia	January 1985	5 × 4	6/8
IT3	Domegliara Veneto, Italy	2002	June Lady	February 1992	5 × 3.2	5/8
FR1	Balandran, France	2001	Gladys	February 1996	6 × 4	27/8
FR2	Balandran. France	2001	Fairlane	February 1988	6 × 1.5	31/8
FR3	Balandran, France	2002	Sweet Fire	February 2000	4 × 1.5	19/7

Table 2
Treatments applied in field experiments in Spain (SP), Italy (IT), and France (FR)

Control group	Treatment No.	Trial	Year	No. of applications		Description
				Bloom	Pre-harvest	
Biological	T1 ($CB10^6 \times 4$)	SP1,IT1,IT2,IT3, FR1,FR2,FR3	2001, 2002	2	2	10^6 fresh *E. nigrum* conidia ml^{-1}
	T2 ($CB10^7 \times 4$)	IT3	2002	2	2	10^7 fresh *E. nigrum* conidia ml^{-1}
	T3 ($CBF10^6 \times 4$)	IT3	2002	2	2	10^6 formulated *E. nigrum* conidia ml^{-1}
	T4 ($CB10^6 \times 2$)	SP1,IT1,IT2	2001	0	2	10^6 fresh *E. nigrum* conidia ml^{-1}
	T5 ($CB10^6 \times 2$)	SP1,IT1,IT2	2001	2	0	10^6 fresh *E. nigrum* conidia ml^{-1}
Chemical	T6 (CQ)	SP1,IT1,IT2,IT3,FR3	2001, 2002	2	1	0.38 mg a.i. ml^{-1} triforine (at bloom in SP1,IT1,IT2, and at preharvest in SP1), 0.02 mg a.i. ml^{-1} cyproconazole (at bloom in IT3,FR3, and at preharvest in IT1,IT2, IT3), 0.125 mg a.i. ml^{-1} tebuconazole (at preharvest in FR3)
		FR1,FR2	2001	1	2	0.02 mg a.i. ml^{-1} cyproconazole (at bloom), 0.125 mg a.i. ml^{-1} tebuconazole (1st preharvest), 0.125 mg a.i. ml^{-1} iprodione (2nd preharvest)
Integrated	T7 (CI1)	IT1,IT2,IT3,FR3	2001, 2002	2	2	At bloom:0.38 mg a.i. ml^{-1} triforine (IT1,IT2) or 0.02 mg a.i. ml^{-1} cyproconazole (IT3,FR3)/ at preharvest: 10^6 fresh *E. nigrum* conidia ml^{-1}
	T8 (CI2)	IT1,IT2,IT3,FR3	2001, 2002	2	1	At bloom: 10^6 fresh *E. nigrum* conidia ml^{-1}/at preharvest: 0.02 mg a.i. ml^{-1} cyproconazole (IT1,IT2,IT3) or 0.125 mg a.i. ml^{-1} tebuconazole (FR3)
Other	T9 (NT)	SP1,IT1,IT2,IT3, FR1,FR2,FR3	2001, 2002	0	0	Untreated

dry weight of substrate with a viability higher than 80% (Larena et al., 2004).

The formulated product (F) was obtained as follows. After centrifugation, fresh conidia (obtained as described above) were resuspended in sterile distilled water added into the centrifuge tubes. Conidial suspensions were then filtered through 1 μm filter paper in a Büchner funnel, and the resulting conidial paste was introduced in a fluid bed-dryer 350s (Burkard Manufacturing, Hertfordshire, UK) two times for 20 min each time at the highest air flow rate and at 40 °C. Under these conditions samples were disaggregated (Larena et al., 2003). Dried conidia were thoroughly mixed with additives 10 min before their use in the field. Additives were Tween 20 (2 ml L^{-1}), glycerol (1 ml L^{-1}), polyethylene glycol 300 (PEG300) (1 ml L^{-1}), and KCl (0.2 g L^{-1}). Viability of *E. nigrum* conidia was higher than 80%.

Fungicides used were triforine 19% P/V (Saprol, BASF Española, S.A., Barcelona, Spain) in trial SP1, cyproconazole 10% [WG] (ATEMI 10 PEPITE 10%, Syngenta, Italy) and triforine 19% P/V (Saprol, BASF, Italy) in trials IT1, IT2, and IT3, and iprodione 43.2% [SC] (ROVRAL Aquaflow, Aventis, France) in trials FR1, FR2, and FR3. Orchards received cultural and other crop protection practices common in each location.

One hundred asymptomatic fruits per sample unit were randomly picked by hand at commercial harvest date for each location. These fruits were placed in five packing trays, each containing 20 fruits. All of them were placed in a box and stored at 20 °C and 85% RH for 7 days when percentage of brown rot was visually recorded. The presence of conidia sporulating on lesions were necessary to confirm that symptomatic fruits were infected with *Monilinia* spp. In addition, in 2001 isolations of fungi growing in the lesions were made in potato dextrose agar (Difco, Detroit, MI USA) amended with 0.5 g L^{-1} streptomycin sulphate. Species of *Monilinia* were identified following the description given in De Cal and Melgarejo (1999).

Results from each trial of the field were analysed independently by contrast with the *F* test at significance levels of 0.1, 0.05, and 0.01 (Snedecor and Cochran, 1980).

3. Results

The percentages of brown rot after 7 days of harvest are presented in Table 3. Disease level on untreated trees ranged from 58 to 84%. The highest levels of brown rot were recorded in Spain (80%) and Italy (84%).

Table 3
Percentage of decayed fruit by *Monilinia* spp. in trials carried out in different countries during 2001 and 2002 after different treatments[a]

Treatment[b]	Spain	Italy			France		
	SP1	IT1	IT2	IT3	FR1	FR2	FR3
	2001	2001	2001	2002	2001	2001	2002
T1 ($CB10^6 \times 4$)	46 ± 10	39 ± 3	37 ± 4	74 ± 8	64 ± 2	68 ± 3	61 ± 8
T2 ($CB10^7 \times 4$)	—	—	—	72 ± 6	—	—	—
T3 ($CBF10^6 \times 4$)	—	—	—	70 ± 3	—	—	—
T4 ($CB10^6 \times 2$)	77 ± 8	59 ± 3	54 ± 15	—	—	—	—
T5 ($CB10^6 \times 2$)	72 ± 11	61 ± 4	57 ± 5	—	—	—	—
T6 (CQ)	59 ± 10	29 ± 2	28 ± 6	69 ± 2	34 ± 2	44 ± 6	36 ± 5
T7 (CI1)	—	42 ± 1	40 ± 7	66 ± 3	—	—	70 ± 12
T8 (CI2)	—	28 ± 1	29 ± 2	57 ± 7	—	—	327 ± 7
T9 (NT)	80 ± 6	64 ± 2	58 ± 11	84 ± 4	67 ± 4	62 ± 5	74 ± 8

[a] Data are the mean of four replicates ± standard error of the mean.
[b] See Table 2 for details of treatments.

Table 4
Contrast analysis of different methods of control of *Monilinia* spp. in field experiments carried out in Spain, Italy, and France in 2001 and 2002[a]

Treatment[b]	Spain	Italy			France		
	SP1	IT1	IT2	IT3	FR1	FR2	FR3
	2001	2001	2001	2002	2001	2001	2002
$CB(10^6 \times 4)$ vs $CB(10^6 \times 2)$	**	**	*	—	—	—	—
$CB(10^6 \times 4)$ vs NT	**	**	*	NS	NS	NS	**
$CB(10^6 \times 2)$ vs NT	NS	NS	NS	—	—	—	—
$CBF(10^6 \times 4)$ vs NT	—	—	—	**	—	—	—
$CB(10^7 \times 4)$ vs NT	—	—	—	*	—	—	—
CQ vs NT	NS	**	**	**	**	**	**
CQ vs $CB(10^6 \times 4)$	NS	**	NS	NS	**	**	**
CI vs NT	—	**	**	**	—	—	**
CI vs CQ	—	*	NS	NS	—	—	NS
CI vs $CB(10^6 \times 4)$	—	NS	NS	**	—	—	NS
CI1 vs CI2	—	**	NS	NS	—	—	**

[a] *F test significant at $P = 0.1$. **F test significant at $P = 0.05$. NS, not signigicant; —, not tested.
[b] See Table 1 for detail of treatments. CB, biological treatments; $CB(10^6 \times 4)$, biological treatments applied four times at 10^6 conidia ml^{-1}; $CB(10^6 \times 2)$, biological treatments applied twice at 10^6 conidia ml^{-1}; NT, untreated; CBF, formulated cells of *E. nigrum*; $CB(10^7 \times 4)$, biological treatments applied four times at 10^7 conidia ml^{-1}; CQ, chemical treatments; CI, integrated treatments.

Monilinia laxa (85–90%) and *M. fructigena* (10–15%) were isolated and identified as the cause of brown rot in peaches from orchards located in Spain, and Italy in 2001. In addition, the quarantine species *M. fructicola* was isolated and identified in peaches from France in 2001.

Table 4 shows the contrast analysis of different methods used of control (CB, biological; CQ, chemical; and CI, integrated) made by grouping the corresponding groups of treatments in the seven trials (Table 2).

When data from biological treatments applied four times were compared with the untreated control significant differences were observed in four out of seven trials (FR1, FR2, and IT3 resulted not significant) (Table 3). In trial IT3 formulation *F* at 10^6 conidia ml^{-1} (T3) or a dose of 10^7 conidia ml^{-1} (T2) applied four times reduced disease. However, no significant differences were observed when treatments were applied only two times.

When chemical treatments were compared with the untreated control significant differences were observed in all the trials except in SP1, while significant differences were obtained in the trials carried out in France and in IT1 when were compared with biological treatments (Table 3).

A comparison of integrated treatments with the untreated control resulted in significant differences in all the tested four trials (Table 3). Integrated control did not differ significantly either from chemical control, except for IT1 (Table 3), or for biological control, except for IT3. Significant differences were obtained when comparing integrated treatments T8 ($2\times N10^6/1\times Q$) with T7 ($2\times Q/2\times EN10^6$) in IT1 and FR3.

4. Discussion

Previous field experiments showed the potential of *E. nigrum* for biocontrol peach twig blight caused by *M. laxa* (Madrigal et al., 1994; Melgarejo et al., 1986; Pascual et al., 1996). The potential for reduction of brown rot

of peach fruits was further investigated in laboratory experiments by application of *E. nigrum* conidia at postharvest to peaches artificially inoculated with *M. laxa* (Larena et al., 2003). However, no experiment has been reported on the reduction of brown rot after harvest with application of antagonists in the field. The results of the present study show that applications of *E. nigrum* in the orchard (at bloom and preharvest) significantly reduced brown rot of peaches after harvest in five trials out of the seven tested.

Different factors were considered in *E. nigrum* biological treatments to trees: (i) number and timing of application, (ii) application of fresh or formulated cells, and (iii) dose of application. Results indicated that four applications of *E. nigrum* are needed to obtain reduction of disease (Table 4). No significant reduction was obtained when *E. nigrum* was applied only two times, either at bloom or at preharvest. In addition, significant differences were obtained when biological treatments applied four times were compared with biological treatments applied two times in all trials (Table 4). Higher number of treatments resulted also in greater reduction of twig blight in previous experiments (Madrigal et al., 1994). Treatments applied at bloom together with preharvest treatments 15–7 days before harvest are necessary. This fact could be a consequence of epidemiology of disease. Studies carried out in peach orchards in Georgia (USA) demonstrated a relationship of the incidence of latent infection of immature fruits by *M. fructicola* to brown rot at harvest and postharvest (Emery et al., 2000). The study also showed that fruit was more susceptible to be infected by the pathogen at bloom, at the onset of pit hardening, and between 7 and 12 days before harvest, and that the infections remain latent until harvest. Further studies carried out in prune orchards in California (USA), showed the importance of latent infections by *M. fructicola* occurring after bloom on brown rot at harvest and postharvest (Luo and Michailides, 2001a,b). The importance of reducing latent infection between bloom and preharvest was considered and it was shown to be very useful in determining risk analysis in a decision support system for brown rot disease management of prune (Luo and Michailides, 2001b). In our case, it is important to understand the seasonal pattern of fruit susceptibility to latent infection in order to develop a control strategy for brown rot. This should imply application of treatments, either biological or chemical, more than three or four times. Further studies in that direction are in progress.

A solid-state fermentation process producing large quantities of fresh conidia of *E. nigrum* was developed recently in our laboratory (Larena et al., 2004). Experiments carried out here demonstrated that field application of these fresh conidia reduced brown rot of peaches at postharvest at some extent. Enhanced efficiency of formulated biological control agents have been reported (Rhodes, 1993; Vidhyasekaran et al., 1997). Although only one trial was made here, a reduction of disease was obtained after application of a formulation of *E. nigrum* fresh conidia, despite the high pressure of disease (84%). This promising result encourage to continue formulating fresh cells of *E. nigrum* in order to improve the efficiency of disease control.

Increasing the dose of application of biocontrol agents resulted in some cases in a better control of diseases (Campbell, 1989; Cook and Baker, 1983). Although only one trial was made, reduction of disease was obtained with 10^7 conidia ml^{-1}, comparing to 10^6 conidia ml^{-1} in IT3 (Tables 2 and 3).

Chemical control resulted significantly better than biological control in trials carried out in France (Table 4). *M. fructicola* was isolated from rotten peaches in orchards of France in 2001 (Lichou et al., 2002a,b). *M. fructicola* is a more virulent species compared to *M. laxa* or *M. fructigena* (Byrde and Willetts, 1977), and this could be the cause of the worst control obtained with *E. nigrum* in some French experiments. *M. fructicola* is a quarantine organism in Europe (European and Mediterranean Plant Protection Organization, 1992) and measures have been taken to prevent more foci of *M. fructicola*. In an usual case, given in Mediterranean countries infection of peaches is caused by *M. laxa* or *M. fructigena*.

Integrated control is a good alternative to chemical control to reduce brown rot of peaches since the total fungicide application is reduced: the same reduction of disease was obtained with three applications of fungicides vs with two applications of *E. nigrum* plus one application of fungicides. In the cases in which integrated treatment T8 resulted better than integrated treatment T7 (FR3 and IT1) chemical treatments (which were applied before harvest) were essential to reduce disease. In the other two trials biological control worked well, and similar efficacy was obtained with either biological, chemical or integrated control. Previous experiments showed that combinations of the fungicide captan and *E. nigrum* did not improve control of peach twig blight when compared to that obtained with *E. nigrum* or captan alone, except in one of four trials (Madrigal et al., 1994). Nevertheless, in any case integrated treatments of *E. nigrum* with fungicides have advantages over the application of chemical alone: the total fungicide application is reduced.

This work demonstrated that *E. nigrum* applications alone or in combination with fungicides in field treatments to peach trees reduced brown rot at postharvest. A further reduction of disease may be possible by a better knowledge of epidemiology of disease to determine the best moment of treatment application, by using an improved formulation of the biological product, and by combining with postharvest treatments. Studies are in progress in these directions.

Acknowledgments

This work was supported by the European Commission project QoL-PL-1999-01065 and the AGL2002-4396-CO2 project of Plan Nacional de I+D+I, Ministerio de Educación y Ciencia, Spain. We thank all the growers for their support and collaboration.

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