

# Sensitivity of *Monilinia fructicola* from Spanish peach orchards to thiophanate-methyl, iprodione, and cyproconazole: fitness analysis and competitiveness

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**Abstract** Benzimidazoles, dicarboximides and demethylation inhibitors are the main group of fungicides used to control brown rot in Spain. The causal agents of brown rot in peach (*Prunus persica* (L.) Batsch) orchards in Spain are *Monilinia laxa*, *M. fructigena*, and *M. fructicola*. The dynamics of fungicide sensitivity and fitness of *M. fructicola* population, the most recent species causing brown rot in the Ebro Valley (Lleida, Spain), were characterized by determining their resistance, fitness, and in vitro competitiveness to thiophanate-methyl (TM), iprodione (I), and cyproconazole (CPZ) in field isolates recovered over the 2006–2010 period. We found that: (a) more than 95 % of the *M. fructicola* isolates are high TM-resistant, (b) more than 50 % of the *M. fructicola* isolates are I-resistant and these frequencies of occurrence did not change during our 5-year survey, and (c) a few CPZ-resistant isolates have been also detected in population since 2008. We identified five different fungicide-resistant (R) and/or fungicide-sensitive (S) phenotypes and found that our study population contains multiple fungicide-resistant isolates. Moreover, these fungicide-resistant isolates display high parasitic fitness on fruit and flowers and high competitiveness. These findings suggest that the TM, I, and CPZ resistance of *M. fructicola* isolates could be contributing as another factor on changing the frequency of occurrence of the three *Monilinia* species in the Ebro Valley.

**Keywords** Fungicide resistance · *M. fructicola* · Fitness · Phenotype characterization · Species displacement

## Introduction

Brown rot caused by *Monilinia* spp. is one of the most economically important fungal diseases of peaches. In Spain, its incidence may be as high as 80 % when the climatic conditions are favourable for disease development, especially in those orchards which grow late-ripening varieties (Gell et al. 2008). The application of fungicides is a leading stratagem for controlling brown rot, and fungicides are usually applied mainly on pre-harvest 3 to 5 times during each growing season in Spanish peach orchards, especially at preharvest (Usall et al. 2010).

Until 2006, 85–90 % of the brown rot in Spain was caused by *M. laxa* (Aderh et Rulh) Honey and the remaining 10–15 % was caused by *M. fructigena* Honey in Whetzel (Larena and Melgarejo 1996). In 2006, a third *Monilinia* spp., *M. fructicola* (G. Winter) Honey, was detected in peach orchards in the Ebro Valley, Lleida, Spain, (De Cal et al. 2009). Since its detection in 2006, *M. fructicola* has displaced *M. fructigena* and co-existed with the same relative frequency as *M. laxa* (Villarino et al. 2013). The relative frequency of *M. fructicola* was increased along six years in postharvest brown rot fruit, latent infections, pruned branches, and on mummified fruit sampled on the trees; while the frequency of occurrence of *M. laxa* in commercial orchards is progressively declining in 7-day-old

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harvested fruit with brown rot (Villarino et al. 2013). *M. fructicola* isolates were the most pathogenic on nectarine fruit, and showed the greatest morphological differences from *M. laxa* and *M. fructigena* (Villarino et al. unpublished). *M. fructicola* grows faster, sporulates more abundantly, and is better able to facilitate its dispersal than either *M. fructigena* or *M. laxa* (Byrde and Willetts 1977). Furthermore, the pseudosclerotial mummified fruit from which apothecia with ascospores are produced could act as an occasional source of primary *M. fructicola* inoculum in stone fruit orchards (Holtz et al. 1998). The presence of the teleomorphic stage in *M. fructicola* could also increase its evolutionary potential because of outcrossing and the generation of new genotypic combinations (Free et al. 1996). *M. fructicola* ascospores have not yet been found in any European peach orchards (EFSA 2011), although we have previously reported that the *M. fructicola* population in the Sudanell orchard (Lleida, Spain) would potentially have a teleomorphic stage (Villarino et al. 2012). In another recent work of our group we have identified six vegetative compatibility groups (VCGs) in our study population and we were able to produce apothecia of our *M. fructicola* population in the laboratory, whereas no different VCGs or apothecia were described for *M. laxa* (De Cal et al. 2014). Finally, the potential of *M. fructicola* to rapidly develop resistance to fungicides (Michailides et al. 1987) led us also to consider the resistance as another factor involved on the species displacement.

MBC-resistant *M. fructicola* isolates have been found in countries where the fungus has been long established, such as the USA (Ma et al. 2003; Michailides et al. 1987; Zhu et al. 2010), Australia (Penrose 1990), New Zealand (Elmer and Gaunt 1994; Sanoamuang and Gaunt 1995) and Brazil (May-de Mio et al. 2011). The mechanism of MBC action (including thiophanate-methyl (TM)) is to disrupt mitosis and cell division, and the Fungicide Resistance Action Committee (FRAC) has classified the TM as a fungicide class at high risk to develop resistance (Fishel and Dewdney 2006). In the same way, the FRAC has classified the DFC fungicide class at high to medium risk to develop resistance (Fishel and Dewdney 2006). Iprodione (I) is a DCF, and has been used for controlling brown rot for almost two decades in the USA and resistance has not yet been reported in field *M. fructicola* isolates (Ma et al. 2006; Yoshimura et al. 2004). However, reduced I sensitivity has been reported

in *M. fructicola* isolates in New Zealand (Elmer and Gaunt 1994) and in Australia (Wherrett et al. 2001). Cyproconazole (CPZ) is a DMI-fungicide that acts inhibiting sterol biosynthesis in membranes, and it is considered by FRAC as a medium risk one (Fishel and Dewdney 2006), DMI-resistance has been already reported in *M. fructicola* populations from the USA, (Luo and Schnabel 2008; Schnabel et al. 2004; Villani and Cox 2011; Chen et al. 2013) and in Brazil (May-De Mio et al. 2011).

Mutations that are associated with fungicide resistance may concomitantly exert deleterious pleiotropic effects and fitness costs, which only become apparent in the absence of fungicide selection pressure (Schoustra et al. 2005). The evolution of fungicide resistance in fungal populations is largely dependent on the fitness of those strains which can influence the competition dynamics between fungicide-resistant and fungicide-sensitive strains. The parasitic and saprophytic fitness of a fungus can be defined as the survival and reproductive success of an allele, individual, or group (Pringle and Taylor 2002), and fitness has important implications for disease management (Peever and Milgroom 1995). Nevertheless, fungicide resistance strains might achieve a stable frequency of occurrence due to a process known as fitness compensation, that has been shown to involve second site mutations rather than reversion to fungicide sensitivity, resulting in resistant strains not only growing well in the presence of the fungicide but also in its absence (Schoustra et al. 2005).

The resistance of *M. fructicola* populations has been stable for several decades and benzimidazole-resistant and benzimidazole-sensitive *M. fructicola* isolates are reported to be equally fit and equally parasitic (Yoshimura et al. 2004). In contrast, reduced fitness of DCF-resistant isolates has been reported for several ascomycetes, including *M. fructicola* (Raposo et al. 1995; Sanoamuang and Gaunt 1995). Cox et al. (2007) reported that the fitness of propiconazole-resistant *M. fructicola* isolates is the same as that of propiconazole-sensitive isolates at the time of their collection. They also reported that fitness of the propiconazole-resistant isolates decreases and that propiconazole resistance becomes unstable depending on storage conservation and over consecutive transfers to petri dishes.

In a recent study that included four *M. fructicola* isolates of our population, it was demonstrated that mutations leading to high levels of resistance to MBC

fungicides were present in Europe (Weger et al. 2011). Despite the fact that resistance to fungicides has been also detected in *M. laxa* populations in Europe (Thomidis et al. 2009; Malandrakis et al. 2012, 2013), we have not detected resistance to MBCs, DFCs or DMIs until 2006, in our *M. laxa* study population. Our hypothesis is based on the possibility of a differential behaviour of *Monilinia* spp. against these groups of fungicides that could be favouring as another factor on changing the establishment of *M. fructicola* rather than *M. laxa* or *M. fructigena* in our field population.

The aim of this work was to study the dynamics of fungicide sensitivity and fitness of *M. fructicola* field isolates in Spanish peach orchards. The specific objectives of this investigation were to: (i) survey these *M. fructicola* isolates for their frequency of occurrence and sensitivity and/or resistance to thiophanate-methyl, iprodione, and cyproconazole, and (ii) assess the fitness and competitiveness of fungicide-sensitive and fungicide-resistant *M. fructicola* isolates in order to determine if fungicide resistance could be indicated as another factor advantaging *M. fructicola* over *M. laxa* and *M. fructigena* and to eventually develop appropriate disease management strategies for *M. fructicola* in the Ebro Valley, Spain.

## Materials and methods

### Characterization of fungicide resistance

The investigation comprised 535 *M. fructicola* field isolates. The 535 isolates were randomly collected from 109 orchards sited on the left margin of the Ebro. Orchards were sampled in different peach growth stages (*M. fructicola* was isolated from cankers, mummies, pruning branches, fruit and flower latent infections, harvested and post harvested fruit) and 3–10 isolates were collected in each orchard. Samplings were carried out in 2006, 2007, 2008, 2009 and 2010. These commercial orchards were annually treated with thiophanate-methyl, iprodione and cyproconazole along these 5 years, individually or in combinations with others sharing or not the same chemical group, following national and European recommendations. Each isolate was placed consecutively for 7 days at 20–25 °C on potato dextrose agar (PDA) (Accumedia Manufacturers, Lansing, MI, USA) with 0.5 % streptomycin sulphate until a pure culture from a single colony. Each isolate

was also stored in glass assay tubes on potato dextrose agar (PDA) (Accumedia Manufacturers, Lansing, MI, USA) at 4 °C and/or in Eppendorf microtubes with 20 % glycerol at –80 °C. The isolates were identified using their morphological characteristics (De Cal and Melgarejo 1999) and a polymerase chain reaction (PCR)-based assay with specific primers *IColaS* (GAGACGCACACAGAGTCAG) and *IColaAS* (GAGACGCACATAGCATTGG) that were developed in our laboratory (Gell et al. 2007). For the PCR-based identification assay, DNA was extracted using a DNA extraction kit (Qiagen GmbH, Hilden, Germany) and PCR conditions were identical to those that were described by Gell et al. (Gell et al. 2007).

Resistance to benzimidazoles, DCFs, and/or DMIs in 535 *M. fructicola* isolates were tested using 2 and 6 µg ml<sup>-1</sup> TM (Thiophanate-methyl Technical, Bayer Crop Science, France, Villefranche sur Saône, France), 5 µg ml<sup>-1</sup> I (Iprodione Technical, BASF SE, Limburgerhof, Germany), and 0.3 µg ml<sup>-1</sup> CPZ (Atemi®10; 10 % p/p cyproconazole; Sandoz Agro S.A.E., Barcelona, Spain), respectively. The concentrations of these three fungicides were the same as those that were used to discriminate between sensitive and resistant strains in previous studies that evaluated products sharing the same mechanism of action (Cox et al. 2007; Katan and Shabi 1981; Ma et al. 2003).

Testing for fungicide resistance in the 535 isolates was done in two phases. In the first phase, resistance to the three fungicides was investigated in 235 isolates that were collected from 79 orchards in 2006, 2007, and 2010. Since CPZ resistance was not detected within the 2006 and 2007 isolates, but it was detected in 2010. Thus, we investigated the time of onset of CPZ resistance (discriminate doses 0.3 µg ml<sup>-1</sup>) within 300 isolates that were collected from 30 orchards in 2008 and 2009.

Petri dishes with three compartments were used to test for fungicide resistance. For testing for M-resistance, compartments contained PDA amended with 2 µg ml<sup>-1</sup> TM, PDA amended with 6 µg ml<sup>-1</sup> TM, and unamended PDA. Petri dishes with three compartments were also used for testing for I and CPZ resistance. Again, compartments contained PDA amended with 5 µg ml<sup>-1</sup> I, PDA amended with 0.3 µg ml<sup>-1</sup> CPZ and unamended PDA. For each fungicide and isolate, 5-mm diameter mycelial plugs that were removed from the edge of growing colonies of each isolate, were placed on three fungicide-amended PDA plates and then

incubated for 7 days at 25 °C in the dark. The growth of each isolate on fungicide-amended PDA was visually scored on a continuous scale from 0 to 3 ( $X=0$ =no growth of the isolate on the amended PDA;  $0<X\leq 1$ =growth of the isolate on the fungicide-amended PDA was under or equal to one-third of the growth of the same isolate on unamended PDA;  $1<X\leq 2$ =growth of the isolate on the fungicide-amended PDA was between one-third to two-thirds of the growth of the same isolate on unamended PDA; and  $2<X\leq 3$ =growth of the isolate on the fungicide-amended PDA over two-thirds to the growth of the same isolate on unamended PDA). Then these values were transformed into percentages of relative growth by dividing the visual scale value on amended PDA by that on unamended PDA and multiplied by 100. Three plates for each concentration, fungicide and isolate were used. The complete assay was repeated twice.

## Fungicide sensitivity

The range of sensitivity to the three fungicides was determined from growth response curves of 21 selected *M. fructicola* isolates to increasing fungicide concentrations (Table 1). The 21 isolates that were used in this assay comprised 2 isolates with the  $TM^{S1}I^S CPZ^S$  phenotype, 1 isolate with the  $TM^{S1}I^R CPZ^S$  phenotype, 5 isolates with the  $TM^{R1}I^R CPZ^S$  phenotype, 6 isolates with the  $TM^{R1}I^S CPZ^S$  phenotype, and 7 isolates with the  $TM^{R1}I^R CPZ^R$  phenotype (Table 1). Three replicates per isolate, fungicide and dose were measured. The growth response curves were constructed by growing each isolate in individual wells of a microplate that contained potato dextrose broth (PDB; Difco Laboratories, MI, USA) (1000 conidia/well in a final volume of 100  $\mu$ l) and increasing concentrations of TM (0, 100, 150, 200, 250, and 300  $\mu$ g  $ml^{-1}$ ), I (0, 1, 1.5, 2, 2.5, 3, 4, and

**Table 1** Half maximal effective concentration ( $EC_{50}$ ) of 21 *Monilinia fructicola* isolates with different fungicide resistance or sensitivity phenotypes

Isolate name (Year of collection)	Phenotype <sup>a</sup>	Thiophanate-methyl $EC_{50}$ ( $\mu$ g $ml^{-1}$ )	Iprodione $EC_{50}$ ( $\mu$ g $ml^{-1}$ )	Cyproconazole $EC_{50}$ ( $\mu$ g $ml^{-1}$ )
Z1C3R11T (2006)	$TM^{R1}I^R CPZ^S$	>300	2.28	0.09
SUD32-1 (2006)	$TM^{R1}I^R CPZ^S$	>300	2.32	0.07
Z5C4M2 (2006)	$TM^{R1}I^R CPZ^S$	>300	2.14	0.13
ALFIL7DAC6 (2007)	$TM^{R1}I^R CPZ^S$	>300	2.28	0.1
ECOL2010FLR5.1 (2010)	$TM^{R1}I^R CPZ^R$	N.D	3.21	0.2
Z3C32R (2007)	$TM^{R1}I^R CPZ^S$	>300	2.35	0.09
SUD53-1-2 (2006)	$TM^{R1}I^S CPZ^S$	>300	1.47	0.09
ALF.57-5 (2006)	$TM^{R1}I^S CPZ^S$	>300	1.64	0.11
Z4C3R6 (2006)	$TM^{R1}I^S CPZ^S$	>300	1.5	0.1
Z1C8R5T (2006)	$TM^{R1}I^S CPZ^S$	>300	1.56	0.095
ALF.83.3.2 (2006)	$TM^{R1}I^S CPZ^S$	>300	1.28	0.105
Z5C171R (2010)	$TM^{R1}I^S CPZ^S$	>300	1.21	0.09
SUD 111-5 (2006)	$TM^{S1}I^S CPZ^S$	<2	1.41	0.105
SUD. 65-1 (2006)	$TM^{S1}I^S CPZ^S$	<2	1.35	0.125
ALF2010COS1R3 (2010)	$TM^{R1}I^R CPZ^R$	>300	3.47	0.3
ALF2010COS2R7 (2010)	$TM^{R1}I^R CPZ^R$	>300	2.1	0.28
SUD2010IL7DAC9R8.2 (2010)	$TM^{R1}I^R CPZ^R$	>300	2.31	0.19
Z5C132R (2010)	$TM^{R1}I^R CPZ^R$	>300	2.47	0.3
Z5C191R (2010)	$TM^{R1}I^R CPZ^R$	>300	3.09	0.35
Z5C201R (2010)	$TM^{R1}I^R CPZ^R$	>300	3.55	0.32
ECOL2010FLR7.1 (2010)	$TM^{S1}I^R CPZ^S$	<2	2.46	0.11

<sup>a</sup> TM=Potato dextrose agar (PDA) 2  $\mu$ g  $ml^{-1}$  thiophanate-methyl amended; I=PDA 5  $\mu$ g  $ml^{-1}$  iprodione amended; CPZ=PDA 0.3  $\mu$ g  $ml^{-1}$  cyproconazole amended; <sup>R</sup>=Resistant; <sup>S</sup>=Sensitive

5  $\mu\text{g ml}^{-1}$ ), or CPZ (0, 0.1, 0.2, 0.3, 0.4, and 0.5  $\mu\text{g ml}^{-1}$ ) for 46 h at 22 °C in the dark. The growth of each isolate at each fungicide concentration was determined spectrophotometrically by measuring the absorbance of the conidial-containing PDB in each well at 492 nm at the end of the 46-hour incubation. The resultant growth response curves for each fungicide were analysed by the checked method of Raposo et al. (1995) in order to determine the fungicide half maximal effective concentration ( $\text{EC}_{50}$ ). The  $\text{EC}_{50}$  of the growth response curves for each fungicide was determined at least twice.

#### Parasitic fitness in vitro

The parasitic fitness of 33 *M. fruticola* isolates, which we found as having different resistance (R) or sensitivity (S) to each fungicide in the initial screen, was determined recording the incubation and latency periods of each isolate after their inoculation on flowers and fruit. The 33 isolates that were used in this assay included up to 10 isolates representatives from each phenotype (depending on availability) and comprised 2 isolates with the  $\text{TM}^{\text{S}}\text{I}^{\text{S}}\text{CPZ}^{\text{S}}$  phenotype, 1 isolate with the  $\text{TM}^{\text{S}}\text{I}^{\text{R}}\text{CPZ}^{\text{S}}$  phenotype, 10 isolates with the  $\text{TM}^{\text{R}}\text{I}^{\text{R}}\text{CPZ}^{\text{S}}$  phenotype, 10 isolates with the  $\text{TM}^{\text{R}}\text{I}^{\text{S}}\text{CPZ}^{\text{S}}$  phenotype, and 10 isolates with the  $\text{TM}^{\text{R}}\text{I}^{\text{R}}\text{CPZ}^{\text{R}}$  phenotype. The incubation period was the time at which the first symptoms appeared on the flowers and fruit, and the latency period was the time at which the first sporulation occurred on the flowers and fruit.

For determining parasitic fitness of the 33 isolates on flowers, five flowers (replicates) from *cv. Autumn Free* nectarine trees were first inoculated with 3-mm diameter mycelial plugs of each isolate previously grown for a week on a PDA plate. Plugs were obtained using partially-cut 100  $\mu\text{l}$  tips and set on the sepals with the aid of a sterile toothpick. For determining the parasitic fitness on fruit, nectarines (*cv. Nectar Ross*) were first surface-disinfected by immersing each fruit in a 1 % NaOCl solution for 5 min, followed by immersion in 70 % ethanol for one minute, and then finally rinsed twice in sterile distilled water (Sauer and Burroughs 1986). After disinfection, three 5-mm diameter mycelial plugs of each isolate previously grown for a week on a PDA plate were placed on three fruit without wound. Plugs were 100  $\mu\text{l}$  tips made and set on the exocarp of fruit with the aid of a sterile toothpick. Flowers and fruit were incubated in humidity chambers for 7–10 days at

25 °C (100  $\mu\text{E m}^{-2} \text{s}^{-1}$  with a 16-hour photoperiod) at a relative humidity of 100 %. The flowers were examined for blight symptoms under a Wild stereomicroscope (40 $\times$  magnifications) every 6 h for 7 days. Fruit were visually examined for symptoms of brown rot every 6 h for 7 days. The complete assay was repeated twice.

#### Competitiveness in vitro

The competitiveness of 12 *M. fruticola* isolates, which we found as having different fungicide sensitivity in the fungicide sensitivity assay, was determined from the germination and growth abilities of one isolate in the presence of a second one. For this purpose, the 12 different isolates were paired in 10 combinations of dissimilar fungicide-sensitive (Table 2). The 12 isolates that were used in this assay included up to 3 isolates (depending on availability) representatives from each phenotype and comprised 2 isolates with the  $\text{TM}^{\text{S}}\text{I}^{\text{S}}\text{CPZ}^{\text{S}}$  phenotype, 1 isolate with the  $\text{TM}^{\text{S}}\text{I}^{\text{R}}\text{CPZ}^{\text{S}}$  phenotype, 3 isolates with the  $\text{TM}^{\text{R}}\text{I}^{\text{R}}\text{CPZ}^{\text{S}}$  phenotype, 3 isolates with the  $\text{TM}^{\text{R}}\text{I}^{\text{S}}\text{CPZ}^{\text{S}}$  phenotype, and 3 isolates with the  $\text{TM}^{\text{R}}\text{I}^{\text{R}}\text{CPZ}^{\text{R}}$  phenotype.

This assay comprised two different phases, a bioassay and an in vitro plate test. For the bioassay, conidial suspensions of each isolate that contained  $1 \times 10^4$  conidia  $\text{ml}^{-1}$  of Czapek broth solution (Difco<sup>TM</sup> Czapek-Dox Broth; Difco, Detroit, MI, USA) were filtered through glass wool in order to remove any mycelial fragments. Three 100- $\mu\text{l}$  aliquots of 56 mixed conidial suspensions (three replicates per isolate combination) were made by mixing 50  $\mu\text{l}$  of the conidial suspension of each isolate in 56 different paired combinations of isolates on glass slides. These slides were then incubated in humidity chambers for 24 h at 20–25 °C in the dark. At the end of the 24-hour incubation, 25  $\mu\text{l}$  of each 100- $\mu\text{l}$  aliquot, containing two different phenotype conidia, were then recovered from glass slides and spread over the surface of an unamended PDA plate and a PDA plate which was amended with 2  $\mu\text{g ml}^{-1}$  TM, 5  $\mu\text{g ml}^{-1}$  I, or 0.3  $\mu\text{g ml}^{-1}$  CPZ (Table 2). Then plates were incubated for another 48 h at 20–25 °C in the dark. Six plates were evaluated for each isolate (three replicates on amended and three replicates on unamended PDA).

Competitiveness was determined from the number of germinated conidia that finally appeared as colony-forming units (CFU) by the resistant and the sensitive phenotype. The conidia of an isolate with a fungicide-



**Table 2** Combinations of fungicide resistant and sensitive phenotypes of the Spanish population of *Monilinia fructicola* used to determine competitiveness

Combination Number	Phenotype 1 <sup>a</sup>	Phenotype 2 <sup>a</sup>	Selective medium
1	TM <sup>R</sup> I <sup>R</sup> CPZ <sup>R</sup>	TM <sup>R</sup> I <sup>R</sup> CPZ <sup>S</sup>	CPZ
2	TM <sup>R</sup> I <sup>R</sup> CPZ <sup>R</sup>	TM <sup>R</sup> I <sup>S</sup> CPZ <sup>S</sup>	CPZ
3	TM <sup>R</sup> I <sup>R</sup> CPZ <sup>R</sup>	TM <sup>S</sup> I <sup>R</sup> CPZ <sup>S</sup>	M
4	TM <sup>R</sup> I <sup>R</sup> CPZ <sup>R</sup>	TM <sup>S</sup> I <sup>S</sup> CPZ <sup>S</sup>	M
5	TM <sup>R</sup> I <sup>R</sup> CPZ <sup>S</sup>	TM <sup>R</sup> I <sup>S</sup> CPZ <sup>S</sup>	I
6	TM <sup>R</sup> I <sup>R</sup> CPZ <sup>S</sup>	TM <sup>S</sup> I <sup>R</sup> CPZ <sup>S</sup>	M
7	TM <sup>R</sup> I <sup>R</sup> CPZ <sup>S</sup>	TM <sup>S</sup> I <sup>S</sup> CPZ <sup>S</sup>	M
8	TM <sup>R</sup> I <sup>S</sup> CPZ <sup>S</sup>	TM <sup>S</sup> I <sup>R</sup> CPZ <sup>S</sup>	M
9	TM <sup>R</sup> I <sup>S</sup> CPZ <sup>S</sup>	TM <sup>S</sup> I <sup>S</sup> CPZ <sup>S</sup>	M
10	TM <sup>S</sup> I <sup>R</sup> CPZ <sup>S</sup>	TM <sup>S</sup> I <sup>S</sup> CPZ <sup>S</sup>	I

<sup>a</sup> TM=Potato dextrose agar (PDA) 2 µg ml<sup>-1</sup> thiophanate-methyl amended; I=PDA 5 µg ml<sup>-1</sup> iprodione amended; CPZ=PDA 0.3 µg ml<sup>-1</sup> cyproconazole amended; <sup>R</sup>=Resistant; <sup>S</sup>=Sensitive

resistant phenotype were able to germinate and form colonies on the selective medium (TM, I, CPZ respectively). The conidia of an isolate with a fungicide-resistant phenotype or a fungicide-sensitive phenotype were both able to germinate and form colonies on unamended PDA. The most competitive isolate in each isolate pair was deemed as that isolate with the highest number of CFU on the plate containing unamended PDA. The complete assay was repeated twice for each isolate pair.

#### Data analysis

All statistical analyses were done using a computerized statistical program (STATGRAPHICS Centurion XVI, StatPoint Technologies, Warrenton, VA, USA). Statistical significance in all assays was set at 5 %. The frequencies of occurrence were calculated for each fungicide and year of collection and data were analysed by a factorial analysis of variance (ANOVA) with fungicide resistance and year of collection as the factors. Means were compared using the Student-Newman-Keuls (SNK) multiple range test. The frequencies of occurrence were arcsine-square root transformed before the analysis in order to improve the homogeneity of variances by using Bartlett and/or Cochran tests. When results from the second round of replication were the same as those of the first round of replication, the samples (six replicates isolate) were pooled before analysis and the results are reported accordingly.

The incubation and latency periods on the flowers and fruit were analysed using a general linear model (GLM) and the means were compared using the SNK multiple range test. When results from the second round

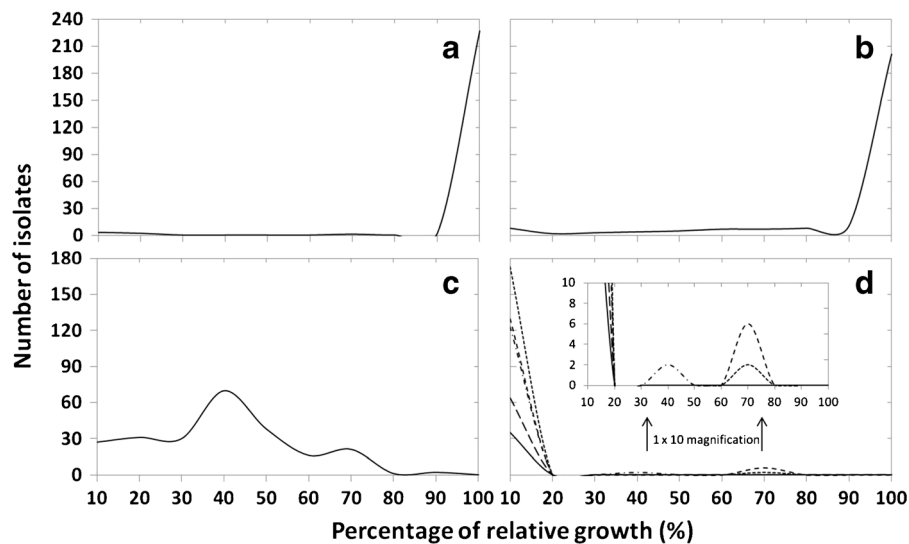
of replication were the same as those of the first round of replication, the samples (six replicates for the fruit-based assay and 10 replicates for the flower-based assay) were pooled before analysis and the results are reported accordingly.

The competitiveness data were analysed by one-way ANOVA, and the means were compared using the SNK range test. The data were log (x+1) transformed before the analysis in order to improve the homogeneity of variances checked by Bartlett and/or Cochran tests. When results from the second round of replication were the same as those of the first round of replication, the samples (six replicates per isolate) were pooled before analysis and the results are reported accordingly.

## Results

### Characterization of fungicide resistance

Either in the case of TM or I, significant differences regarding to sampling years or experiments were not detected on the number of resistant isolates or their resistance levels. Therefore, the 235 isolates were considered together as a single population whose resistance to TM or I is represented in Fig. 1a, b and c. The 235 *M. fructicola* field isolates growth on 2 µg ml<sup>-1</sup> TM-amended PDA was high. We also found that only three of these isolates were completely sensitive to this fungicide and that the growth of more than 225 of the TM-resistant isolates was >95 % (Fig. 1a). When this assay was repeated using 6 µg ml<sup>-1</sup> TM-amended PDA, we found that that nearly all of those isolates that were able to grow on 2 µg ml<sup>-1</sup> TM-amended PDA were also able



**Fig. 1** Growth of *Monilinia fructicola* isolates on (a) 2 µg ml<sup>-1</sup> thiophanate-methyl (b) 6 µg ml<sup>-1</sup> thiophanate-methyl (c) 5 µg ml<sup>-1</sup> iprodione and (d) 0.3 µg ml<sup>-1</sup> cyproconazole-amended potato dextrose agar (PDA). Growth is expressed as a percentage of the isolates growth on PDA (100 %). Data were subjected to arcsine transformation before ANOVA analysis ( $p=0.05$ ) to improve homogeneity of variances. Variances resulted homogeneous according to Bartlett and Cochran ( $p=0.05$ ) variance tests and

to grow on 6 µg ml<sup>-1</sup> TM-amended PDA and just eight isolates were completely sensitive (Fig. 1b). When grown on 5 µg ml<sup>-1</sup> I-amended PDA, we found that this study population comprises three subpopulations of I-resistant isolates represented by three different peaks in Fig. 1c. One subpopulation comprised 30 isolates whose growth was between 20 and 30 % of the growth of isolates on unamended PDA. The second subpopulation, which was the largest one, comprised 70 isolates whose growth is between 61 and 70 % of the growth of isolates on unamended PDA. The third one was formed by 21 isolates whose growth reached up to 70 %.

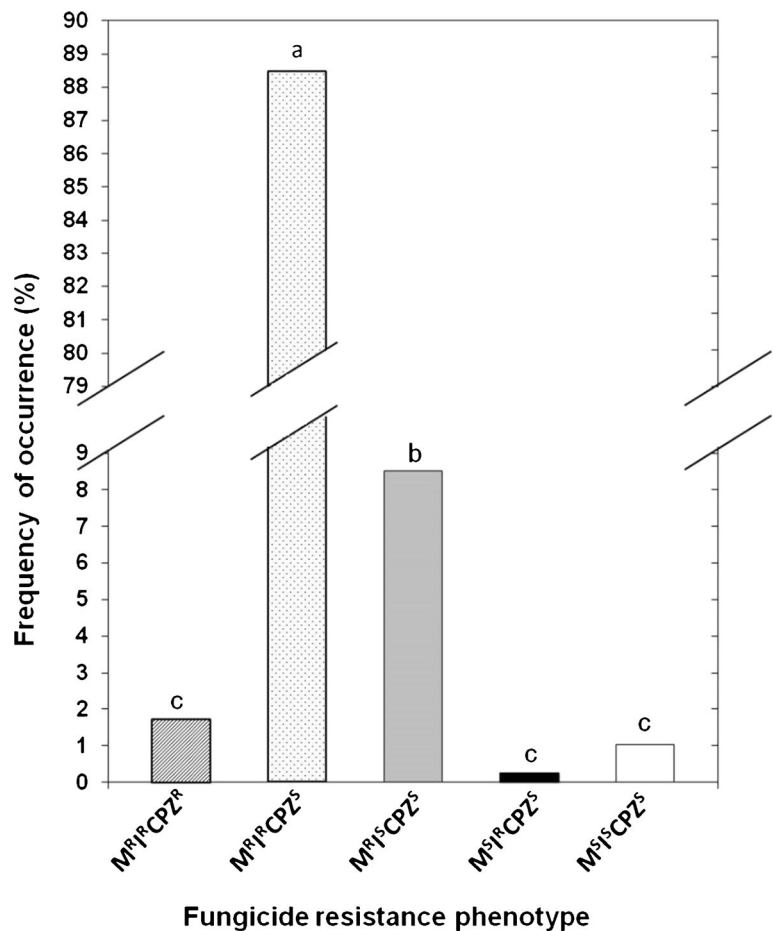
We found that all isolates that were collected in 2006 and 2007 were not able to grow on 0.3 µg ml<sup>-1</sup> CPZ-amended PDA and only seven isolates that were collected in 2010 were resistant to CPZ (Fig. 1d). Specifically, the growth of these CPZ-resistant isolates that were collected in 2010 was around 40 % of the growth of isolates on unamended PDA. In order to determine the first occurrence of CPZ resistance in the population, we then tested 300 isolates that were collected in 2008 and 2009 for their CPZ sensitivity. We also found (a) 2 CPZ-resistant isolates that were collected in 2008 and whose growth is 31–40 % of the growth

experiments were displayed together with six replicates per isolate. There were no significant differences along the years 2006, 2007 and 2010 on percentages of relative growth of 235 isolates represented in panels a) b) and c) at 5 % of significance level. There were significant differences along the years 2006, 2007, 2008, 2009 and 2010 at 5 % of significance level and the isolates are separately represented for each year in figure d).

of isolates on unamended PDA and (b) 2 CPZ-resistant isolates that were collected in 2009 and whose growth is 61–70 % of growth of isolates on unamended PDA in 2009.

Figure 2 summarizes the different frequency of occurrence of those isolates with a fungicide-resistant phenotype among the 235 isolates that were collected in 2006, 2007 and 2010. *M. fructicola* population comprised five subpopulations of isolates with the following phenotypes: TM<sup>R</sup>I<sup>R</sup>CPZ<sup>R</sup>, TM<sup>R</sup>I<sup>R</sup>CPZ<sup>S</sup>, TM<sup>R</sup>I<sup>S</sup>CPZ<sup>S</sup>, TM<sup>S</sup>I<sup>R</sup>CPZ<sup>S</sup> and TM<sup>S</sup>I<sup>S</sup>CPZ<sup>S</sup>. Differences on these phenotypes frequencies of occurrence among years were no detected. Consequently, isolates of all the years were considered as forming each phenotype subpopulation. We found that the most predominant subpopulation was the TM<sup>R</sup>I<sup>R</sup>CPZ<sup>S</sup> phenotype: its frequency of occurrence was 88.48 %, significantly higher than the frequency of the others. We also found that the second most predominant subpopulation was the TM<sup>R</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype: its frequency of occurrence in the population was 8.52 %. We found that the triple-sensitive TM<sup>S</sup>I<sup>S</sup>CPZ<sup>S</sup>, the triple-resistant TM<sup>R</sup>I<sup>R</sup>CPZ<sup>R</sup>, and the I-resistant TM<sup>S</sup>I<sup>R</sup>CPZ<sup>S</sup> phenotypes were rarely found in the population and their respective frequencies of occurrence were less than 2 % in the population studied.

**Fig. 2** *Monilinia fructicola* Spanish population fungicide resistance phenotypes and their frequencies of occurrence. Frequency of phenotype occurrence in population was calculated using 253 isolates that were collected in 2006, 2007, and 2010. Data were subjected to  $\log(x+1)$  transformation before ANOVA analysis ( $p=0.05$ ) to improve homogeneity of variances. Variances resulted homogeneous according to Cochran ( $p=0.05$ ) variance test and experiments were displayed together with 6 replicates per isolate. Values with the same letter in each bar are not significantly different from each other according to the results of the Student-Newman-Keuls multiple range test. TM=  $2 \mu\text{g ml}^{-1}$  thiophanate-methyl; I=  $5 \mu\text{g ml}^{-1}$  iprodione; CPZ=  $0.3 \mu\text{g ml}^{-1}$  cyproconazole; <sup>R</sup>= resistant; <sup>S</sup>=sensitive



TM<sup>S</sup>I<sup>R</sup>CPZ<sup>R</sup>, TM<sup>S</sup>I<sup>S</sup>CPZ<sup>R</sup>, and TM<sup>R</sup>I<sup>S</sup>CPZ<sup>R</sup> phenotypes were not found anywhere.

#### Fungicide sensitivity

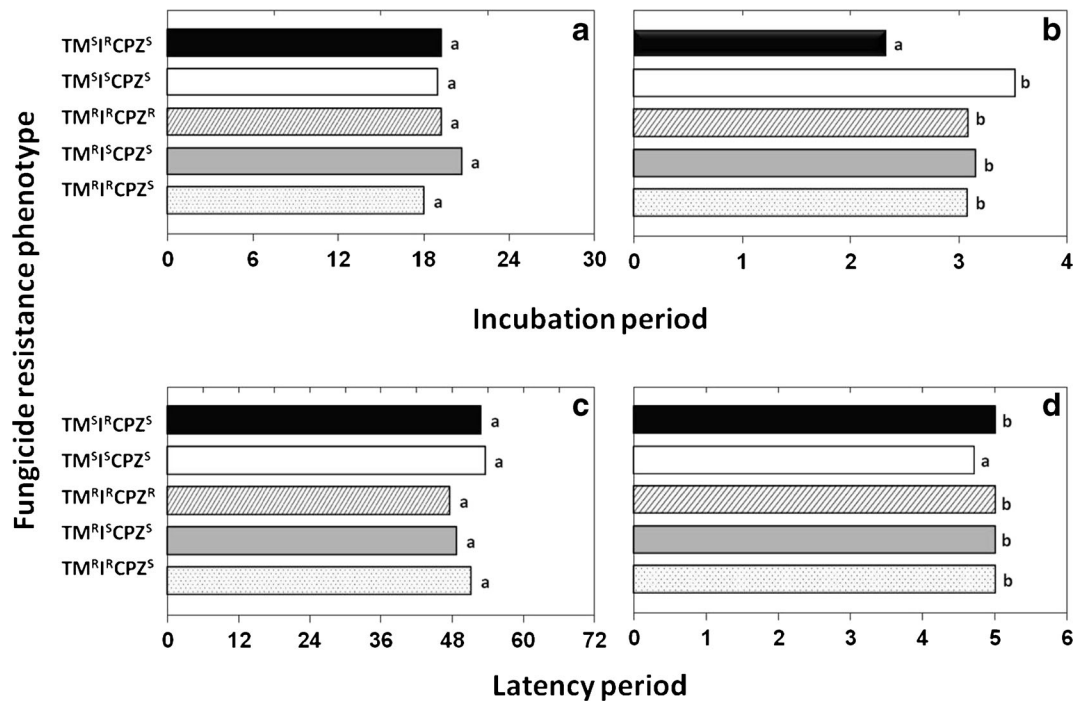
As expected, the EC<sub>50</sub>'s of the growth response curves of the fungicide-resistant isolates were higher than those of the growth response curves of the fungicide-sensitive isolates (Table 1). Specifically, the EC<sub>50</sub>'s of the growth response curves of the I-resistant isolates were between 2.1 and 3.5  $\mu\text{g ml}^{-1}$ , whereas the EC<sub>50</sub>'s of the growth response curves of the I-sensitive isolates were between 1.2 and 1.6  $\mu\text{g ml}^{-1}$  (Table 1). The EC<sub>50</sub>'s of the growth response curves of all CPZ-resistant isolates were between 0.19 and 0.35  $\mu\text{g ml}^{-1}$  whereas the EC<sub>50</sub>'s of the growth response curves of the CPZ-sensitive isolates were between 0.07 and 0.13  $\mu\text{g ml}^{-1}$  (Table 1). The EC<sub>50</sub>'s of the growth response curves from all high TM-resistant isolates were >300  $\mu\text{g ml}^{-1}$ .

#### Parasitic fitness in vitro

We found that the incubation periods on flowers of those isolates with fungicide-sensitive phenotypes were not significantly different from each other (Fig. 3a). Disease symptoms appeared and were microscopically visible on the flowers about 18–20 h after mycelia plugs were placed on them. In contrast, we found significant differences in the incubation periods on fruit among those *M. fructicola* isolates with differing fungicide-sensitive phenotypes (Fig. 3b). The incubation period of isolates with the TM<sup>S</sup>I<sup>R</sup>CPZ<sup>S</sup> phenotype was significantly shorter (2.33 days) than that of isolates with the triple-sensitive TM<sup>S</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype (3.52 days).

We also found that the latency periods on flowers and fruit also differed: sporulation of *M. fructicola* isolates began about 50 h after the mycelia plugs were placed on flowers and no statistically significant differences in the latency periods were recorded (Fig. 3c). On the contrary, we did find differences in the latency period on fruit





**Fig. 3** Parasitic fitness parameters of *Monilinia fructicola* Spanish population represented by (a) Incubation period (hours) of *M. fructicola* phenotypes on the flowers. (b) Incubation period (days) of *M. fructicola* phenotypes on fruit. (c) Latency period (hours) of *M. fructicola* phenotypes on flowers. (d) Latency period (days) of *M. fructicola* on fruit. The 33 isolates that were used in this assay included up to 10 isolates representatives from each phenotype (depending on availability) and comprised 2 isolates with the TM<sup>S</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype, 1 isolate with the TM<sup>S</sup>I<sup>R</sup>CPZ<sup>S</sup> phenotype, 10 isolates with the TM<sup>R</sup>I<sup>R</sup>CPZ<sup>S</sup> phenotype, 10

isolates with the TM<sup>R</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype, and 10 isolates with the TM<sup>R</sup>I<sup>R</sup>CPZ<sup>R</sup> phenotype. Data were subjected to General Linear Model (GLM) analysis ( $p=0.05$ ). Variances resulted homogeneous according to Bartlett and Cochran ( $p=0.05$ ) variance tests and experiments were displayed together with 6 (fruit) and 10 (flowers) replicates per isolate. Values with the same letter in each bar are not significantly different from each other according to the results of the Student-Newman-Keuls multiple range test. TM= 2  $\mu\text{g ml}^{-1}$  thiophanate-methyl; I=5  $\mu\text{g ml}^{-1}$  iprodione; CPZ= 0.3  $\mu\text{g ml}^{-1}$  cyproconazole; R=resistant; S=sensitive

(Fig. 3d): the latency period of isolates with the TM<sup>R</sup>I<sup>R</sup>CPZ<sup>R</sup> phenotype was significantly shorter (4.7 days) than that of isolates with the TM<sup>S</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype (5.0 days).

Competitiveness in vitro

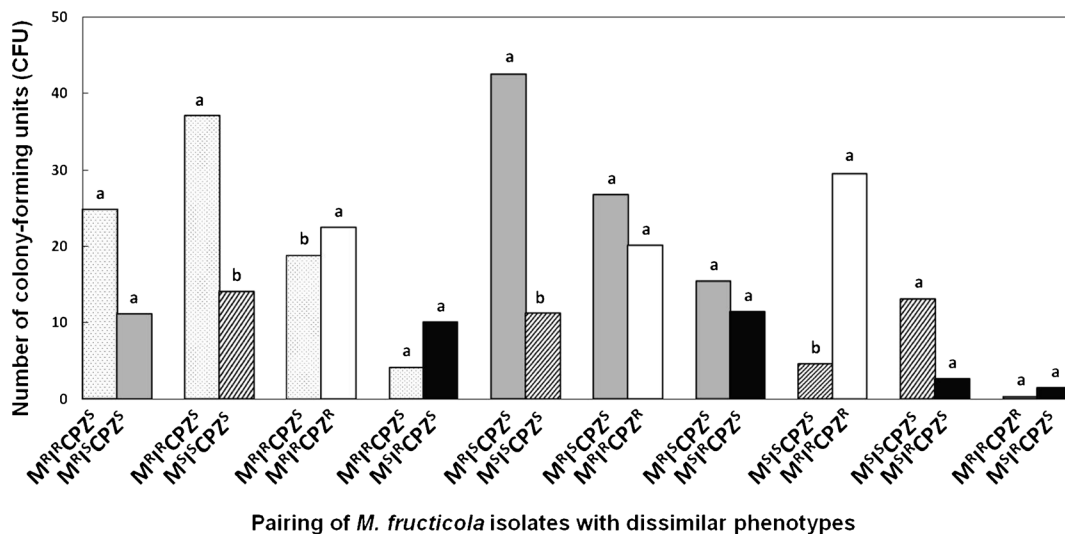
Figure 4 summarizes the results of the competitiveness assays. Phenotypes (X-axis) were compared by their respective ability to germinate (number of colony-forming units (CFU), Y-axis) in the presence of another phenotype in the same plate after a previous 24 h bio-assay. The most competitive phenotype was the one which recorded a higher number of CFU after competition.

We found that all isolates with fungicide-resistant phenotypes were at least as competitive as those isolates with the triple-sensitive TM<sup>S</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype. In

general, the most competitive phenotypes were those with thiophanate-methyl resistance. The single I-resistant TM<sup>S</sup>I<sup>R</sup>CPZ<sup>S</sup> phenotype and the triple-sensitive TM<sup>S</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype were the least competitive isolates of all the isolates.

Discussion

We have previously reported that *M. fructicola* has already displaced *M. fructigena*; and, since 2010, *M. fructicola* co-exists with *M. laxa* at the same frequency of occurrence in peach and nectarine orchards in the Ebro Valley (Villarino et al. 2013). The results of this survey, which comprised 535 *M. fructicola* isolates that were collected from 109 Spanish peach orchards in 2006, 2007, 2008, 2009, and 2010, revealed the presence of *M. fructicola* isolates that were resistant to TM, I



**Fig. 4** Competitiveness between pairings of *Monilinia fructicola* isolates with dissimilar fungicides sensitivity phenotypes. The 12 isolates that were used in this assay included up to 3 isolates (depending on availability) representatives from each phenotype and comprised 2 isolates with the TM<sup>S</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype, 1 isolate with the TM<sup>R</sup>I<sup>R</sup>CPZ<sup>S</sup> phenotype, 3 isolates with the TM<sup>R</sup>I<sup>R</sup>CPZ<sup>S</sup> phenotype, 3 isolates with the TM<sup>R</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype, and 3 isolates with the TM<sup>R</sup>I<sup>R</sup>CPZ<sup>R</sup> phenotype. Data were subjected to log (x+1) transformation before ANOVA analysis ( $p=0.05$ ) to

improve homogeneity of variances. Variances resulted homogeneous according to Bartlett and Cochran ( $p=0.05$ ) variance tests and experiments were displayed together with 6 replicates per isolate. Values with the same letter in each bar are not significantly different from each other according to the results of the Student-Newman-Keuls multiple range test. TM=2  $\mu\text{g ml}^{-1}$  thiophanate-methyl; I=5  $\mu\text{g ml}^{-1}$  iprodione; CPZ=0.3  $\mu\text{g ml}^{-1}$  cyproconazole; R=resistant; S=sensitive

and CPZ and displayed good fitness. These findings suggest that the fungicide resistance of the *M. fructicola* population is co-acting among other factors as an adaptation in the pathogen to change the frequency of occurrence of the three *Monilinia* species, *M. laxa*, *M. fructicola* and *M. fructicola*, in the Ebro Valley, Spain.

In our monitoring of Spanish peach orchards for the presence of *M. fructicola* in the Ebro Valley, which is the main peach growing region in Spain, we did not detect this fungus in the 428 *Monilinia* isolates that we collected between 1998 and 2005 (Gell et al. 2008; Larena and Melgarejo 1996). In contrast, in this investigation, we have characterized the fungicide sensitivity of the founder *M. fructicola* population from the Sudanell orchard, which was the first orchard where *M. fructicola* was detected in 27 isolates which were collected in 2006 (De Cal et al. 2009). We have found that this founder population comprised isolates with the double-resistant TM<sup>R</sup>I<sup>R</sup>CPZ<sup>S</sup> phenotype (85.2 %), isolates with the single-resistant TM<sup>R</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype (7.4 %), and isolates with triple-sensitive TM<sup>S</sup>I<sup>S</sup>CPZ<sup>S</sup> phenotype (7.4 %). We also found that the EC<sub>50</sub> of the growth response curve of the isolates with the TM-resistant

phenotype was >300  $\mu\text{g ml}^{-1}$ . This high EC<sub>50</sub> is in agreement with the high EC<sub>50</sub>'s that have been reported for the growth response curves of other high fungicide-resistant *M. fructicola* populations (Ma et al. 2003; Yoshimura et al. 2004). The EC<sub>50</sub>'s (2.17–2.45  $\mu\text{g ml}^{-1}$ ) of the growth response curve of the I-resistant isolates were slightly lower than those that have been reported in another I-resistant *M. fructicola* population in New Zealand (>3  $\mu\text{g ml}^{-1}$ ) by Elmer and Gaunt (Elmer and Gaunt 1994). The EC<sub>50</sub>'s (0.0095–0.1162  $\mu\text{g ml}^{-1}$ ) of the growth response curve of the CPZ-sensitive isolates were consistent with those that have been reported for CPZ-sensitive *M. fructicola* populations from New York (Wilcox and Burr 1994), South Carolina, and Georgia in the USA (Holb and Schnabel 2007; Schnabel et al. 2004; Villani and Cox 2011); but something differences with that reported by Lichtemberg in Brazil (Lichtemberg et al. 2014).

We found that more than 95 % of the *M. fructicola* isolates were highly TM-resistant and that the frequency of occurrence of these high TM-resistant isolates did not change over the duration of our 5-year survey. The frequency of occurrence of TM-resistant *M. fructicola* isolates shows a qualitative or “single-step” profile of

resistance (Brent and Hollomon 2007). However, high frequency of occurrence of TM-resistant isolates in the population is alarming also because of their high fitness, including the relatively harsh inoculation method we used to induce infection, and also because TM-resistant phenotypes resulted the most competitive ones in competition trials. Furthermore, our results imply that the TM-resistant alleles were present in the founder *M. fruticola* population and these TM-resistant alleles have been maintained and have spread in the population since 2006. Collectively, these findings suggest that, in addition to gene mutation(s) and selection pressure by fungicide application(s), genetic drift due to a founder effect needs to be considered as the third evolutionary force for the presence of TM-resistance in the Spanish *M. fruticola* population. The use of TM-based fungicides (with only two applications per year) is still permitted to control brown rot in Spain (MAGRAMA 2013). Although restricting further use of this fungicide to control brown rot is desirable, this restriction would not necessarily prevent control failures because of the high fitness and stable frequency of occurrence of *M. fruticola* isolates with a multi-fungicide resistant phenotype once qualitative resistance has developed (Brent and Hollomon 2007).

Iprodione use in Spain to control brown rot is two applications/year restricted (MAGRAMA 2013). We found that about a half of the *M. fruticola* isolates were I-resistant at different levels and we found that this frequency of occurrence has not changed during our 5-year survey. The profile of resistance in the Spanish *M. fruticola* population indicates that the I-resistance is quantitative or “two-step” resistance. We found that the frequency of occurrence of I-resistance in our study population is in accordance with the high cost of mutations leading to I-resistance in ascomycetes (Raposo et al. 1995; Sanoamuang and Gaunt 1995). However, the frequency of occurrence of low I-resistant isolates in population is worrying because I-resistance is a new characteristic which was not present in *Monilinia* spp. in the Ebro Valley prior to 2006. Furthermore, our results imply that I-resistant alleles from the source *M. fruticola* population were present in the founder *M. fruticola* population and that these I-resistant alleles have been maintained and have spread since 2006. However, recently has been established that iprodione HR *Botrytis cinerea* isolates did not suffer a competitive disadvantage compared with S isolates (Grabke et al. 2014). Therefore, resistance management programs

should not rely on the exploitation of reduced fitness as a tool to control iprodione-resistant mutants (Grabke et al. 2014).

DMI fungicides such as CPZ are permitted to control brown rot in Spain since 1990 and restricted to four applications per year (MAGRAMA 2013). We did not find CPZ-resistant *M. fruticola* isolates in the first two years of our 5-year survey. Unfortunately, 2–7 CPZ-resistant isolates were detected in our annual study population since 2008, even though frequency of occurrence is still low in population. Indeed, resistance has been found even using a dose that could be considered high for a strong triazole as CPZ is. Anyway, our  $EC_{50}$  results supported that the dose selected was not underestimating resistance in our population, using the discriminate doses ( $0.3 \mu\text{g ml}^{-1}$ ) described by Ma et al. (2003). The CPZ resistance in the Spanish *M. fruticola* population is a recently acquired characteristic and it is probably caused by a mutation in situ or a new introduction, because CPZ resistance was not detected in the founder *M. fruticola* population of 2006. The stability of this CPZ resistance must be further studied.

The frequency of occurrence of TM-resistant and I-resistant *M. fruticola* isolates, and especially those with the  $TM^{R1}I^{R}CPZ^S$  phenotype, which we found was the most competitive phenotype in this investigation, was maintained in our study population during our 5-year survey. Indeed, results of phenotype distribution of population are in accordance to results obtained in competitiveness trials: those isolates that behaved most competitive ( $TM^{R1}I^{R}CPZ^S$ ,  $TM^{R1}I^S CPZ^S$ ,  $TM^{R1}I^{R}CPZ^R$ ) were quantitatively more represented in population.

The high fitness and competitiveness of the fungicide-resistant populations presents serious obstacles for disease control of the Spanish *M. fruticola* population because restricting the use of TM and I fungicides in the stone fruit orchards does not guarantee the return of a fungicide-sensitive population. The high degree of target specificity and the dual modes of action (preventative and curative) of DMIs make DMIs an attractive choice for controlling brown rot in stone fruit orchard and storage facilities in Spain. However, the presence of CPZ-resistant *M. fruticola* isolates indicates that their presence in Spanish peach orchards could progressively increase over time. The application of fungicides to control brown rot should be based on a prediction model that minimizes their use. DMIs only should not be repeatedly applied to the same crop in one season against a high-risk pathogen in areas of high

disease pressure for a particular pathogen. When fungicides are needed to repeatedly applied during the growing season in order to control disease, FRAC has recommended that alternation (block sprays or in sequence) or mixtures with an effective non-cross-resistant fungicide are used. Importantly, these recommendations should be transmitted to growers in order to prevent resistance development in fungal pathogens.

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