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Research Paper

Control of bottom rot in hydroponic lettuce, caused by strains of *Botrytis cinerea* with multiple fungicide resistance

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Summary. For two consecutive growing periods, fungicide-resistant *Botrytis cinerea* strains were detected in high proportions in glasshouse-grown lettuce, but at variable frequencies. Pre-transplanting fungicide sprays applied on two successive occasions reduced disease severity and increased the number of healthy plants without leaving detectable residues above accepted MRLs at harvest. In some instances, the disease was further decreased when pre-transplanting applications were combined with one or two further sprays applied soon after transplanting. The fungicide mixture of fludioxonil + cyprodinil was the most effective against the disease and provided better control of *B. cinerea* isolates *in situ*. These treatments gave satisfactory disease control despite the predominance of multi-fungicide resistant *B. cinerea* populations.

Keywords. Fungicides, grey mould, fludioxonil, cyprodinil, chlorothalonil.

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INTRODUCTION

Bottom rot of butterhead lettuce (*Lactuca sativa* L.) caused by *Botrytis cinerea* Pers. :Fr. is the most common disease problem in hydroponic lettuce production in Greece, during the late autumn to early spring period. Infections can start in nurseries and spread systemically (endophytically) in plants, without early visible symptoms (Sowley *et al.*, 2010). *Botrytis* head rots are less common and, in most cases, follow the appearance of 'tip burn' symptoms, due to inadequate transport of calcium into emerging leaves. These rots can be avoided by keeping calcium in balanced nutrient solutions, using cultivars which are less susceptible to 'tip burn', and manipulating the environment (Morgan, 1999; 2012). Good ventilation practices reducing excess of moisture combined with application of fungicides give adequate control of infections caused by *Botrytis cinerea* (Dik and Wubben, 2007).

In addition to multi-site fungicides such as thiram, compounds with site-specific modes of action against grey mould in lettuce crops are currently registered in Greece. These include anilinopyrimidines (cyprodinil

and pyrimethanil), the phenylpyrrole fludioxonil, the succinate dehydrogenase inhibitor (SDHI) boscalid and the quinone outside inhibitor (QoI) pyraclostrobin. Two commercial fungicide formulations with widespread use against *B. cinerea* in lettuce are Signum® (26.7% boscalid + 6.7% pyraclostrobin; BASF) and Switch® (25% fludioxonil + 37.5% cyprodinil; Syngenta). However, the use of fungicides for *B. cinerea* control in various crops has been associated with the development of fungicide resistance (Hahn, 2014). High levels of resistance against site-specific fungicides are the result of gene mutations at positions encoding their target sites. For example, the point mutations G143A, H272R, and F412S, which lead to changes in the target proteins CytB, SdhB, and Erg27, confer high resistance of the pathogen to, respectively, the QoI, SDHI, and hydroxyanilide fungicide classes (Leroux, 2007).

Multi-drug resistance (MDR) is another mechanism associated with fungicide resistance in *B. cinerea*. This involves mutations leading to over-expression of efflux transporters such as the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS), allowing weak resistance towards fungicides with unrelated modes of action (Kretschmer *et al.*, 2009). Very often, MDR and specific fungicide resistance types are coupled (Leroch *et al.*, 2013; Fernández-Ortuño *et al.*, 2014; Rupp *et al.*, 2016). The presence of *B. cinerea* strains with multiple fungicide resistance to all site-specific classes of fungicides have been reported in different parts of the world, especially for small fruits (Weber, 2011; Amiri *et al.*, 2013; Fernández-Ortuño *et al.*, 2014). Recent surveys made on lettuce crops in Greece and Germany have also demonstrated increasing threats from emergence of multiple fungicide resistance in *B. cinerea* populations (Chatzidimopoulos *et al.*, 2013; Weber and Wichura, 2013).

Current trends in agriculture demand fewer chemical applications, while maintaining profitable high-quality production with low pesticide residues. The limited number of registered fungicide formulations against bottom rot of lettuce forces growers to make repeated seasonal sprays with the one fungicide. Some studies have also shown that pesticide residues are detected in greater amounts in leafy vegetables compared to other crops (Skovgaard *et al.*, 2017). Multiple applications may compromise reduced pesticide strategies, which aim to delay the development of resistance and reduce pesticide residues.

The present study was undertaken: (i) to evaluate the efficacy and timing of applications with current botryticides against multi-resistant *B. cinerea* strains; (ii) to detect and measure possible fungicide residues at harvest; and (iii) to determine effects of different compounds against selected resistant isolates of the pathogen *in situ*.

MATERIALS AND METHODS

Host plant material

The 2-year experiments were carried out in a commercial lettuce glasshouse located at Krokion, Magnesia, Greece. The glasshouse was surrounded by cereal crops and olive trees, which were unlikely to be sources of *B. cinerea* inoculum. Pelletized lettuce seeds (*Lactuca sativa* 'Penelope'; 'butterhead' type, Rijk Zwaan), pre-treated with thiram were used in all tests. Seeding, germination and emergence of nursery plants took place in 4 × 4 cm horticultural cells filled with a peat-based substrate. The young seedlings were transplanted at the 4- to 5-leaf stage, about 5 weeks after sowing, into Hortiplan hydroponic gutters (nutrient film technique, NFT). For plant nutrition, a dense aqueous (bore water) solution was prepared, composed of (mg L^{-1}): Ca 200, Mg 40, K 210, P (PO_4^{3-}) 50, N (NH_4^+) 25, N (NO_3^-) 165, Fe 5, Mn 0.5, Cu 0.1, Zn 0.1, B 0.5, Mo 0.05. The pH of the nutrient solution was maintained between 5.5 and 5.8, and conductivity between 1.5 and 2.0 mS (Resh, 2012). The aqueous solution was supplied to plant every 15 min during daytime.

Experimental design and treatments

Experiments were organized in randomized blocks with three replicates for each treatment. Each plot consisted of 50 plants spaced 20 cm apart in one row. Fungicide applications was carried out using a hand-operated sprayer at 1,120 L ha^{-1} at 10 to 20-day intervals. To minimize the effects on neighbouring treatments, plots were separated with a plastic frame (100 × 50 cm), during the spray applications. The last application was made at least 4 weeks before harvest. Plants sprayed with water were used as experimental controls.

The fungicides used, at the standard recommended labelled rates for vegetable crops, were as follows: chlorothalonil (Daconil® 50 SC, Syngenta Ltd) at 3 mL L^{-1}; fenhexamid (Teldor ® 50 WG, Bayer CropScience) at 1.5 g L^{-1}; boscalid + pyraclostrobin (Signum® 26.7 + 6.7 WG, BASF SE) at 1.5 g L^{-1}; and fludioxonil + cyprodinil (Switch® 25 + 37.5 WG, Syngenta Crop Protection AG) at 0.5 g L^{-1}.

In the first trial (2012-2013 season), lettuce was sown on November 8, 2012, was transplanted into the NFT system on December 7, and was harvested on January 24, 2013. In this trial, the effectiveness of chlorothalonil and the mixture of fludioxonil + cyprodinil was evaluated in two, three or four spray programmes (Table 1). Fungicides were applied on two occasions during the

Table 1. Comparison of fungicide spray programs against bottom rot in hydroponic lettuce (Trial 1).

Applications			Disease		AUDPC	Healthy plants at harvest (%)	Fungicide residues[a] (mg Kg^{-1})	Phenotypes[b] recovered (%)		
Pre-Transplanting (2)	Post-Transplanting (1 or 2)		Severity (%)	Incidence (%)				I	II	W
Fludioxonil+ cyprodinil	-	-	0.6 c[c]	5.3 d	4.19 b	92.67 b	<LoQ[d]	0	0	0
	Fludioxonil+ cyprodinil	-	0.4 c	2.7 d	2.80 b	85 b	Fludioxonil:<LoQ Cyprodinil:0.053	0	0	0
	Fludioxonil+ cyprodinil	Fludioxonil+ cyprodinil	0.1 c	0.7 d	0.66 c	93.67 b	Fludioxonil:0.06 Cyprodinil:0.2	0	0	0
Chlorothalonil	-	-	20.7 a	40.7 b	267.14 a	64.67 ab	<LoQ	33	0	67
	Chlorothalonil	-	14.2 ab	26.0 c	201.86 a	64.67 ab	0.061	50	0	50
	Chlorothalonil	Chlorothalonil	9.2 bc	25.3 c	120.26 a	73.33 b	0.073	75	0	25
Control			21.8 a	55.3 a	261.91 a	37.33 a	-	62	15	23

[a] Maximum residue levels as determined by the European Community. Fludioxonil: 15; Cyprodinil:15; Chlorothalonil: 0.01; Fenhexamid: 40; Boscalid: 30; Pyraclostrobin: 2.
[b] *B. cinerea* phenotypes: I=QoI^{R}Bos^{R}Ani^{R}Ben^{HR}Dic^{MR}, II=Hyd^{R}QoI^{R}Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}, W=Wild type.
[c] Means followed by the same letter in each column do not significantly differ (P = 0.05, Student-Newman-Keuls).
[d] LoQ: limit of quantification (0.005 mg Kg^{-1}).

nursery stage (November 19, December 1), followed by one or two more applications after transplanting (December 13 and 24).

In the second trial (2013–2014 season), lettuce was sown on October 17, 2013, transplanted on November 23, and harvested on January 27, 2014. Following the results from the first trial, the addition of fungicides from unrelated chemical groups was also evaluated in two-, three- or four-spray programmes. Two basal applications were carried out during the nursery stage (October 27 and November 17) with either chlorothalonil or the fludioxonil + cyprodinil mixture. After transplanting, one (December 6) or two (December 30) more applications were made with either fenhexamid, fludioxonil + cyprodinil, boscalid + pyraclostrobin or chlorothalonil, in alternating applications (Table 2).

Efficacy of the fungicide programmes was evaluated at harvest by recording the number of healthy plants from each plot. The disease incidence and severity were also recorded every week by counting the number and estimating the proportion (%) lesion area on plants infected by *B. cinerea* in each treatment. The sample size for all assessments was 50 plants per plot. At the end of each trial, the area under the disease progress curve (AUDPC) was calculated, based on the formula:

$$AUDPC = \sum_{i=1}^{N_i-1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i)$$

where t = the time of each assessment; y = the percent disease severity at each assessment; and n = the number of assessments.

Fungicide residues on lettuce heads were determined at harvest, in randomly collected samples of three plants from each treatment. Following EU directions, highly sensitive and selective multi residue methods were used to detect multiple fungicides (Waziha *et al.*, 2018). Chlorothalonil and fenhexamid residues were determined using gas chromatography with an electron capture detector. Boscalid, pyraclostrobin, cyprodinil and fludioxonil were analyzed using liquid chromatography with tandem mass spectrometry, following acetonitrile extraction/partitioning. Analyses were carried out at the Cadmion accredited Analytical Laboratory, 202 00 Kiato Korinthia, Greece.

Airborne inoculum monitoring

Portable air samplers (Burkard Manufacturing Co Ltd) were used, containing 9 cm Petri plates with selective medium (Edwards and Seddon, 2001), as slightly modified by Chatzidimopoulos *et al.* (2014b). The medium was enriched with a discriminatory concentration of each fungicide and was used to entrap *B. cinerea* propagules in the air. Only fungicide-resistant isolates were able to grow on these media. The different fungicides were dissolved in dimethyl sulphoxide and were added to different plates with the selective medium. The final con-

Table 2. Comparison of fungicide spray programs against bottom rot caused by *Botrytis cinerea* in hydroponic lettuce (Trial 2).

Applications			Disease		AUDPC	Healthy plants at harvest (%)	Fungicide residues[a] (mg Kg^{-1})	Phenotypes[b] recovered (%)		
Pre-Transplanting (2)	Post-Transplanting (1 or 2)		Severity (%)	Incidence (%)				I	II	W
Fludioxonil+ cyprodinil	-	-	0.8 b[c]	2.67 b	7.35 b	90.00 a	<LoQ[d]	0	0	100
	Fludioxonil+ cyprodinil		1.2 b	7.33 b	9.72 b	97.33 a	<LoQ	0	100	0
	Fenhexamid	-	0.7 b	0.67 b	11.64 b	95.33 a	<LoQ	0	100	0
	Boscalid+ pyraclostrobin	-	0.9 b	2.67 b	9.58 b	96.67 a	<LoQ	50	50	0
	Chlorothalonil	-	1.1 b	2.67 b	15.71 b	98.00 a	<LoQ	100	0	0
	Fenhexamid	Fludioxonil+ cyprodinil	2.4 b	10.67 b	21.92 b	94.00 a	Fludioxonil:0.43 Cyprodinil:0.74 Fenhexamid<LoQ	25	75	0
	Fludioxonil+ cyprodinil	Fenhexamid	0.9 b	1.33 b	11.93 b	92.00 a	Fludioxonil <LoQ Cyprodinil<LoQ Fenhexamid:5.95	0	100	0
Chlorothalonil	-	-	1.7 b	3.33 b	23.94 b	83.33 a	<LoQ	100	0	0
	Chlorothalonil	-	0.6 b	1.33 b	6.26 b	96.67 a	<LoQ	0	0	0
	Fenhexamid	-	2.1 b	4.67 b	33.22 b	89.33 a	<LoQ	25	75	0
	Boscalid+ pyraclostrobin	-	0.8 b	1.33 b	13.38 b	95.33 a	<LoQ	75	25	0
	Fludioxonil+ cyprodinil	-	1.7 b	2.00 b	26.84 b	93.33 a	<LoQ	0	75	25
	Fenhexamid	Fludioxonil+ cyprodinil	3.0 b	4.67 b	45.34 b	89.33 a	Fludioxonil:0.43 Cyprodinil:0.74 Fenhexamid<LoQ	0	0	0
	Fludioxonil+ cyprodinil	Fenhexamid	1.6 b	3.33 b	20.62 b	92.00 a	Fludioxonil <LoQ Cyprodinil<LoQ Fenhexamid:5.95	0	100	0
Control			14.2 a	28 a	182.58 a	88.00 a		60	20	20

[a] Maximum residue levels as determined by the European Community. Fludioxonil: 15; Cyprodinil:15; Chlorothalonil: 0.01; Fenhexamid: 40; Boscalid: 30; Pyraclostrobin: 2.
[b] *B. cinerea* phenotypes: I=QoI^{R}Bos^{R}Ani^{R}Ben^{HR}Dic^{MR}, II=Hyd^{R}QoI^{R}Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}, W=Wild type.
[c] Means followed by the same letter in each column do not significantly differ (P = 0.05, Student-Newman-Keuls).
[d] LoQ: limit of quantification (0.005 mg Kg^{-1}).

centration of the solvent in the growth medium did not exceed 1%. The discriminatory doses used were: 1 mg L^{-1} fenhexamid from the hydroxyanilide (Hyd) class; 10 mg L^{-1} pyraclostrobin from QoI class (strobilurins) plus 100 mg L^{-1} salicylhydroxamic acid; 10 mg L^{-1} boscalid (Bos) from the SDHI class (carboxamides); 10 mg L^{-1} cyprodinil from the anilinopyrimidine (Ani) class; 1 mg L^{-1} fludioxonil from the phenylpyrrole (Phen) class, and 3 mg L^{-1} iprodione from dicarboximide (Dic) class. Plates containing no fungicide were used as experimental controls.

The air samplers were operated simultaneously for 60 min near midday once every 10 d during the experimental periods. To avoid the formation of holes in the medium, the agar layer inside each Petri dish was thick (each dish contained approximately 20 mL of medium). Typical *B. cinerea* sporulating colonies developed on the sampling media, following 6 d of incubation at 20°C in the dark. The number of colonies on each fungicide-containing medium was expressed as the proportion of the total number of colonies on the control plates.

Isolation of the pathogen and definition of the resistance phenotype

Botrytis cinerea was isolated from plants bearing lesions at the stem bases, during the harvest. Infected

plant tissues were transferred in separate moist polyethylene bags to the laboratory and stored at room temperature for 24 h. From each sample a single isolate was made onto sterilized potato dextrose agar (PDA) media by slight touching a flamed wire loop onto a freshly sporulating *Botrytis* lesion. In order to identify the phenotype, each isolate was tested for sensitivity response to the fungicides fenhexamid, chlorothalonil, pyraclostrobin, boscalid, cyprodinil, fludioxonil, carbendazim (50% WP, Cequisa SA) or iprodione, with the point inoculation method (Chatzidimopoulos *et al*., 2013). The same procedure was followed to determine the phenotype of airborne trapped inocula after 7 d of growth in plates containing *B. cinerea* selective medium, in a sample of 100 collected colony forming units (CFUs). The samples were collected at random from control plates and plates amended with fungicides, among different sampling dates.

In situ *pathogenicity assays*

Lettuce plants were grown in 9 cm diam. plastic pots containing peat substrate, in a growth chamber (Sanyo MLR-350HT) with 10 h light period at 18°C, until the 14th true leaf unfolded. Leaf blades (approx. 4 × 4 cm) were excised from the upper half of each plant and immersed in aqueous fungicide suspensions at the same rates used in the field trials. Control leaf blades were immersed in sterilized water. The leaves were allowed to dry for 30–40 min and were then placed in Petri dishes containing water agar (1.5%) with adaxial surfaces uppermost. Three *B. cinerea* isolates, each from the three dominant phenotypes in the glasshouse, were used as inoculum. Mycelium discs (5 mm diam.) were removed from the periphery of 3-d-old colonies grown on PDA, and were aseptically placed upside down over the leaf blades. The Petri dishes with the inoculated leaves were then placed in the growth chamber conditions described above. After 72 h incubation, the mean diameters of the lesion on each leaf blade (minus the 5 mm of mycelial plug) was determined using a measuring rod. Three replicates per isolate/fungicide treatment were made.

Data analyses

Data from the glasshouse trials were analysed by one-way ANOVA and Student-Newman-Keuls test. Tukey's Honestly Significant Difference test was used to assess differences of mean values from the pathogenicity trials. To meet the assumptions of ANOVA, percentage and count values were logarithmically transformed to base 10 where necessary. P-values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using ARM software (Revision 2017.4, Gylling Data Management, Inc.).

RESULTS

Control of disease

In the untreated control lettuce plants, small, hardly visible, brown lesions appeared at the base of the petioles of the bottom leaves, 14 d after transplanting. *Botrytis cinerea* invaded the basal stems *via* the senescent cotyledons or leaf petioles within 3 weeks after transplanting, at the 13 true unfolded leaf growth stage. Early assessments at 1 to 3 weeks after transplanting indicated that when the infection was initiated at an earlier stage of development, the disease progressed more rapidly and eventually the plant stem bases rotted within 10 to 14 d. All the plants which showed early symptoms of infections within this period eventually rotted. The overall disease severity ranged from 0.11 to 0.3 %, and incidence from 2 to 8 %, in both trials at 14 d after transplanting. When the infection was initiated at a later stage, the stem rot progressed very slowly. The disease was more severe in the first trial than the second. By the time of the last assessment at harvest, disease severity and incidence were up to 21.8 % and 55.3 % for trial 1 and up to 14.2 % and 28 % for trial 2 (Tables 1 and 2).

In first trial, two pre-transplanting applications of fludioxonil + cyprodinil reduced disease incidence and severity compared with unsprayed plants (Table 1). Disease control was further improved when one or two more applications were made after transplanting. The AUDPC values for these treatments were very low ranging from 0.66 to 4.19 and a significantly increased number of healthy plants was observed at harvest compared to the untreated control. Chlorothalonil, although providing some control of the disease, was the least effective fungicide. Although disease incidence and severity incidence were reduced, the mean AUDPC values were high, ranging from 120.26 to 267.14 for all treatments, and these were not significantly different from the untreated control. However, the programme with two post-transplanting sprays of chlorothalonil significantly increased the number of healthy plants at harvest compared to the untreated control. With the exception of post-transplanting applications with chlorothalonil, no fungicide residues exceeding the maximum residue level (MRL) defined by the European Community (EC) were detected at harvest. These are 0.01 mg kg^{-1} for chlorothalonil, 15 mg kg^{-1} for fludioxonil and 15 mg kg^{-1} for cyprodinil. For the pre-transplanting applications alone, the residues at

harvest were below the adopted analytical reporting limits of quantification (LoQs) of 0.005 mg kg^{-1} (Table 1).

In the second trial, with lower disease pressure, two applications of fungicide at the pre-transplanting stage, with either fludioxonil + cyprodinil or chlorothalonil, decreased disease incidence and severity. One or two more post-transplanting fungicide applications with alternating treatments did not improve disease control (Table 2). All the treatments were of high efficacy, and the disease progress (AUDPC) was significantly reduced compared to the untreated control (Table 2). Although increased numbers of healthy plants were observed from most treatments compared to the untreated control, the differences between treatments were not statistically significant. With the exception of two cases (four-spray programmes), in which the fungicides fenhexamid and fludioxonil + cyprodinil were applied at 27 d before harvest, no fungicide residues were detected. Residues of fenhexamid were 5.95 mg kg^{-1} and of fludioxonil + cyprodinil were 0.43 + 0.74 mg kg^{-1}. However, the residue amounts were much less than the European MRLs, at 40 mg kg^{-1} of fenhexamid, 15 mg kg^{-1} of fludioxonil and 15 mg kg^{-1} of cyprodinil (Table 2).

Fungicide resistant airborne inoculum

Fungicide resistant *B. cinerea* inocula in the air of the lettuce glasshouse were detected at all the sampling dates, for both of the trials. The numbers of trapped CFUs in each of the six fungicide-amended substrates are shown in Figure 1. From the beginning until the end of the experimental periods, iprodione-, pyraclostrobin- and cyprodinil-resistant CFUs were detected at frequencies comparable to the CFUs trapped in control plates. CFUs resistant to fenhexamid (Fen^R) were trapped on all sampling dates but at variable frequencies. An increase of the Fen^R population was observed during the second trial. However, the total numbers of CFUs trapped in the media from the mid-December to mid-January were generally low. Boscalid resistant (Bos^R) inocula were detected at variable frequency during both trials. The Bos^R populations reached peaks in the middle of both growing periods. By contrast, with the exception of the late sampling dates during the second trial, fludioxonil resistant ($Phen^{MR}$) CFUs were rarely trapped (Figure 1).

Approximately 90% of the isolates recovered from the control plates exhibited multiple resistance to fungicides (Figure 2). The three prevalent resistant phenotypes in decreased frequency were: $QoI^RBos^RAni^R$-$Ben^{HR}Dic^{MR}$ (phenotype I; 57% frequency), Hyd^RQoI^R-$Bos^RAni^RPhen^{MR}Ben^{HR}Dic^{MR}$ (phenotype II; 33%) and wild type (phenotype W; 10%). Only phenotype-II CFUs were detected in media amended with fenhexamid or fludioxonil (Figure 2). In media amended with pyraclostrobin, cyprodinil, iprodione and boscalid, the most

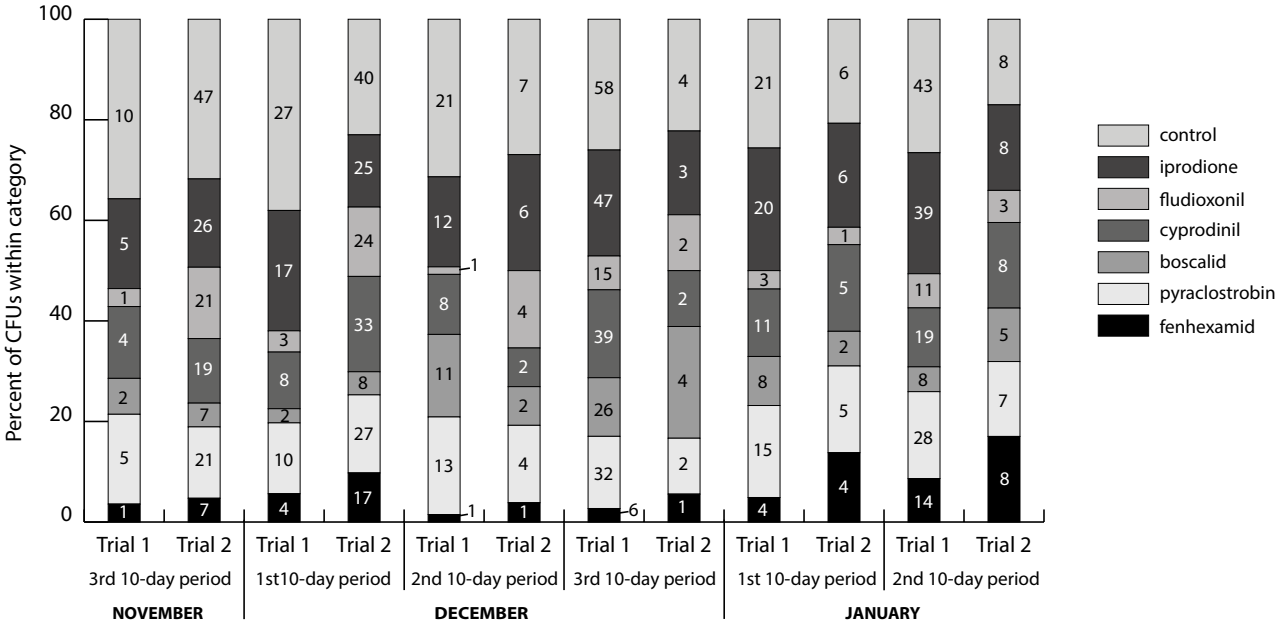

Figure 1. Number of airborne *B. cinerea* conidia trapped in different fungicide amended media during 2012-13 (Trial 1) and 2013-14 (Trial 2) growing periods. The number of CFUs on fungicide enriched selective media are indicated in each bar and expressed as % of the total number of CFUs on all the plates including controls (top bars). The discriminatory concentrations used were: 1 mg L^{-1} fenhexamid, 10 mg L^{-1} pyraclostrobin + 100 mg L^{-1} SHAM, 10 mg L^{-1} boscalid, 10 mg L^{-1} cyprodinil, 1 mg L^{-1} fludioxonil and 3 mg L^{-1} iprodione.

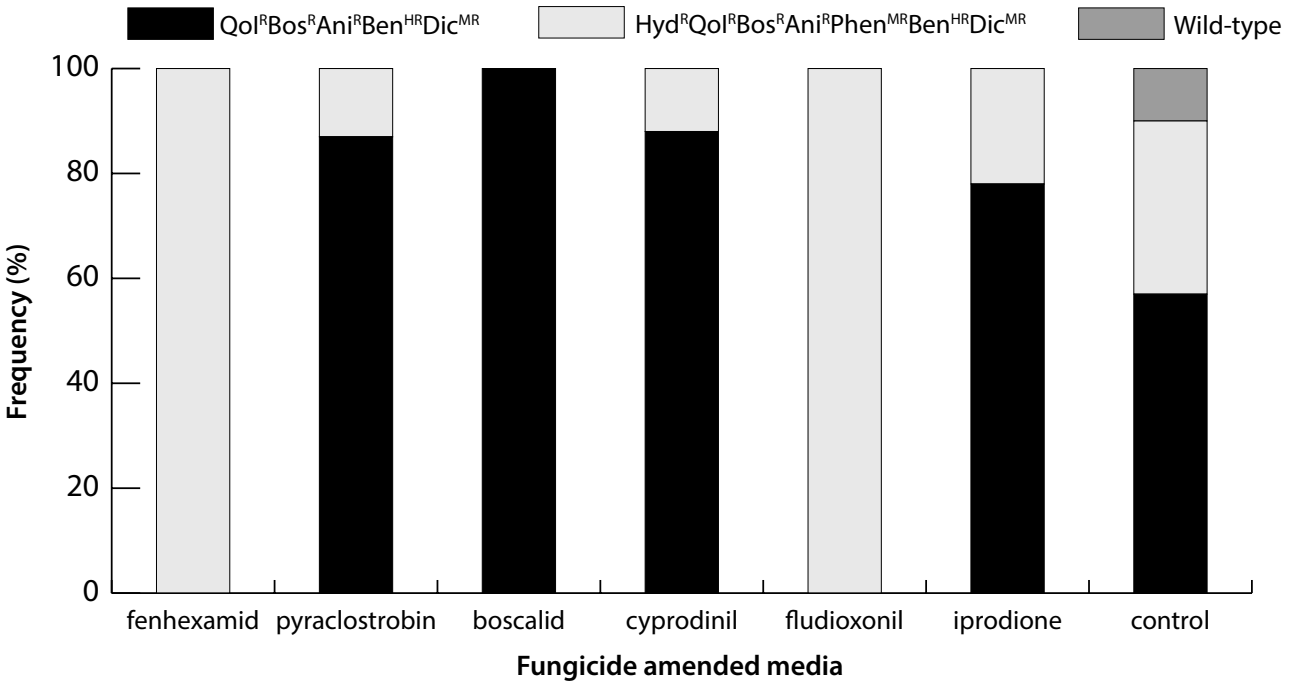

Figure 2. *Botrytis cinerea* phenotypes recovered from fungicide amended selective media. The sensitivity response of each CFU to the discriminatory doses of the fungicides, was examined by the point inoculation method.

prevalent phenotype was type I at frequencies ranging from 78 to 100%. CFUs of the phenotype II were detected at lower frequencies (12 to 22%) on media containing pyraclostrobin, cyprodinil and iprodione (Figure 2). Those three were the only phenotypes detected on all media tested.

Detection of fungicide resistant strains in diseased plants

All the isolates recovered from infected plants were classified in the following three phenotypes: QoI^{R}-$Bos^{R}Ani^{R}Ben^{HR}Dic^{MR}$ (phenotype I; 58%), $Hyd^{R}QoI^{R}$-$Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}$ (phenotype II; 24%) and wild-type (phenotype W; 18%). The multiple resistant phenotype II was detected in plants treated at least once after transplanting with either fenhexamid, boscalid + pyraclostrobin or fludioxonil + cyprodinil (Table 2). This phenotype was not detected in plants treated only with chlorothalonil. On the other hand, phenotype I prevailed by 60% in the isolations made from the infected plants in the controls (Table 2). Phenotype-I strains were also most frequently detected in treatments where the QoI fungicide was included. No diseased plants were observed from the spray programmes with the mixture of fludioxonil + cyprodinil in the first trial (Tables 1 and 2).

Pathogenicity of fungicide resistant isolates in situ

Fenhexamid, pyraclostrobin, boscalid and cyprodinil failed to inhibit the development of *B. cinerea* lesions *in vitro* when the isolate used in inoculations was characterized as resistant to the respective fungicide. No differences were observed in lesion size compared to controls (Table 3). In contrast, fludioxonil was more effective, even against the isolate CR-32 which was characterized as moderately resistant to this fungicide. Chlorothalonil gave variable effects against the isolates with multiple resistances to other fungicides (Table 3). The mean lesion sizes ranged from 2.6 to 12.2 mm, although these strains had previously been characterized as sensitive *in vitro* to chlorothalonil.

DISCUSSION

Selection of resistant individuals in fungal populations subjected to selective pressure due to fungicides is an evolutionary mechanism that promotes advantageous genotypes (Walker *et al.*, 2013). In the present study, during a 2-year monitoring schedule in a glasshouse, multiple resistant isolates were detected after two to four fungicide applications per year. In addition, the phenotypic characterization of the isolates obtained in this

Table 3. *In situ* lesion development on lettuce leaves treated with fungicides and inoculated with multiple resistant isolates of *Botrytis cinerea*.

Isolate[a]	Size of lesion (mm)						
	Fenhexamid (1.5 g)[b]	Pyraclostrobin (0.4 g)	Boscalid (0.8 g)	Cyprodinil (0.375 g)	Fludioxonil (0.5 g)	Chlorothalonil (2.5 mL)	Control
C-01 (Type I)	0.0 **A**a (S)[d]	18.0 **BC**b (R)	27.0 **C**b (R)	15.4 **B**b (R)	4.6 **A**a (S)	3.4 **A**a (S)	20.0 **BC**b
CR-32 (Type II)	21.0 **BC**b[c] (R)	19.8 **BC**b (R)	24.2 **C**b (R)	18.4 **BC**b (R)	10.9 **A**b (MR)	12.2 **B**b (S)	23.8 **C**b
A-56 (Wild type)	3.0 **A**a (S)	4.6 **A**a (S)	0.0 **A**a (S)	5.4 **A**a (S)	2.6 **A**a (S)	2.6 **A**a (S)	15.4 **B**a

[a] Phenotype: **C-01**=QoI^{R}Bos^{R}Ani^{R}Ben^{HR}Dic^{MR}; **CR-32**=Hyd^{R}QoI^{R}Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}; **A-56**=wild-type.
[b] Fungicide treatments (concentration per L). The fungicide formulations used were: fenhexamid as Teldor 50 WG (Bayer CropScience); pyraclostrobin as F500 25 EC (Syngenta Ltd); boscalid as 510F 50 WG (BASF SE); cyprodinil as Chorus 50 WG (Syngenta Crop Protection AG); fludioxonil as Geoxe 50 WG (Syngenta Crop Protection AG); chlorothalonil as Daconil 500 SC (Syngenta Ltd).
[c] Numbers followed by the same bold upper-case letters in rows and low-case letters in columns do not differ significantly according to Tukey's HSD post hoc test; $P = 0.05$.
[d] Sensitivity group: R=resistant, MR=moderately resistant, S=sensitive.

study, and previous genetic analyses, showed that several resistance alleles to different fungicide molecules were selected, due to fungicide pressure (Chatzidimopoulos *et al*., 2013; Chatzidimopoulos *et al*., 2014a; Chatzidimopoulos *et al*., 2014b). Furthermore, next generation sequencing data revealed that an underlying MDR mechanism was also present in these strains (Chatzidimopoulos *et al*., 2016). The presence of such strains in an isolated area after repeated use of fungicides suggests that a stepwise accumulation of resistances occurred over time, and that no pathogen migration took place from neighbouring crops, as has occurred in other cases (Rupp *et al*., 2016).

Different strategies have been adopted to avoid or reduce the risks of production losses due to fungicide resistance. These have included applications only in pre-formulated or tank fungicide mixes, or in rotations with effective non-cross-resistant fungicides, preferably multi-site inhibitors with low risk and limited numbers of treatments (Brent, 2012). In the present study, despite the existence of multi-resistant airborne inoculum, all the application programs improved disease control and left fungicide residues below the defined European Community MRLs. Few multi-site inhibitors with activity against *B. cinerea* are now available. Use of chlorothalonil, a multi-site inhibitor that is still available, is restricted to certain crops due to deposition of undesirable residues. However, when applied in this study on lettuce in the nursery, satisfactory disease control was achieved without detectable fungicide residues at harvest. Applications with chlorothalonil-based fungicides after transplanting should be avoided, however, since there is then risk of the remaining residues on lettuce being above the accepted LoQ limits. Due to recent decision of the European Standing Committee (SCoPAFF) against the renewal of chlorothalonil registration in EU countries, growers are likely to have (from spring 2020) one fewer vital tool to combat fungicide resistance.

Recent surveys made on lettuce revealed infections by *B. cinerea* strains that are multi-resistant to most available botryticides (Chatzidimopoulos *et al*., 2013; Weber and Wichura, 2013). By using an air sampler with a selective medium, as proposed by Edwards and Seddon (2001), modified and enriched with appropriate doses of fungicides (Chatzidimopoulos *et al*., 2014b)2014b, we revealed the prevalence of *B. cinerea* resistant strains in the air of the glasshouse throughout two experimental periods. Most trapped isolates exhibited multiple resistances to specific fungicides. High degrees of resistance to carbendazim (benzimidazole class) and moderate resistance to iprodione (dicarboximide class) were always present, even when neither of these fungicides was included in the spray programmes. Two dominant resistant phenotypes (QoI^{R}Bos^{R}Ani^{R}Ben^{HR}Dic^{mR} and Hyd^{R}QoI^{R}Bos^{R}Ani^{R}Phen^{MR}Ben^{HR}Dic^{MR}) were detected in the air of the experimental glasshouse and isolated from infected plants. These phenotypes constituted 84% of the total *Botrytis* population in the glasshouse, and had also been isolated from diseased plants originating from the same site in previous years (Chatzidimopoulos *et al*., 2013).

Recent studies have shown that in the absence of fungicide selection pressure, resistance to fenhexamid (Billard *et al*., 2012), cyprodinil (Bardas *et al*., 2008) or boscalid (Veloukas *et al*., 2014) may entail a fitness costs in *B. cinerea*. However, Rupp *et al*. (2016) con-

cluded that multiple resistant strains are likely to possess high fitness in the field, and that they are essentially immune to sprays with any of the current botryticides. In the present study, significant decreases of the airborne resistant populations were observed at the beginning of each growing period. Moreover, no strains highly resistant to fludioxonil have been detected in the field, although this phenylpyrrole fungicide has been used for over two decades (Baroffio *et al*., 2003; Chatzidimopoulos *et al*., 2013). Similarly, Fernández-Ortuño *et al.* (2012) reported the high efficacy of the mixture boscalid + pyraclostrobin against *B. cinerea* in strawberry, even though a resistant population to SDHIs and QoIs was present at high frequency. This information may explain the good efficacy of the fungicides in our field trials. The high frequency of resistant strains within the pathogen population in the atmosphere of the glasshouse may be the consequence of good efficacy of the fungicides against the wild-type *B. cinerea* strains. The present assays have shown that under strong disease pressure, (as for the mycelial plugs used in the *in situ* assays) fungicides lose their efficacy against resistant strains. Under conditions of low disease pressure, however, as for airborne spores in the second trial, the fungicides may retain their efficacy. When conidia of the selected strains were used to check the *in situ* efficacy of the fungicides, lesions were not formed in most cases (unpublished data). The phytoalexin lettucenin A in young plants may act preventively on infections triggered by spores (Bennett *et al*., 1994).

All fungicide applications only at the host nursery stage reduced disease incidence and severity, and increased the number of healthy plants at harvest in a trial with high disease pressure, and to a lesser extent (not significantly) under lower disease pressure. According to Sowley *et al.* (2010), the infections could be initiated at nursery stages and spread systemically throughout the plants without the development of visible symptoms. This disease progress can be arrested by fungicide protection of the young seedlings at the nursery or at early transplanting stages. The pre-transplanting applications provided improved disease control when these were combined with one or two post-transplanting applications. However, the additional applications did not significantly improve the number of healthy plants at harvest. In the second trial, under low disease pressure, the number of healthy plants in control plots was similar with the number of the healthy plants in the treated plots. The initial symptoms of infection were observed at 3 weeks after transplanting, but the disease progressed very slowly because of unfavourable climatic conditions in the glass house. Furthermore, recent studies have shown that lettuce crops become less susceptible to infections as they age (develop thick bases and resistant leaves), and some cultivars, such as iceberg and romaine types, are more prone to infections than others (Shim *et al*., 2014).

The mixture of fludioxonil + cyprodinil was the most effective of the different fungicides tested here against lettuce bottom rot at both rates of application. The high efficacy of this mixture in lettuce crops was also reported by Matheron and Porchas (2008). Kilani and Fillinger (2016) also reported that high resistance to fludioxonil does not exist among *B. cinerea* populations worldwide, and this status is not expected to change in the future. In addition, our results have shown that fludioxonil provided better control of *B. cinerea* resistant isolates *in situ*, in comparison with other fungicides. Similar results were observed by Rupp *et al.* (2016) in *in planta* tomato assays. The inclusion of this compound in the fungicide programmes could explain the effective disease control in field trials.

In conclusion, it was shown that appropriate selection and timing of fungicide sprays is fundamental for control of bottom rot of lettuce grown in hydroponic systems. In a trial with high disease pressure, two fungicide applications during plant growth in the nursery combined with one further application at transplanting, provided the best disease control without leaving detectable fungicide residues at harvest. However, under less disease pressure, the two applications at the nursery stage were enough to protect the plants from bottom rot caused by *B. cinerea*. Fungicide selection was made according to the risk for resistance development. Fludioxonil was the most effective compound against the multiple resistant strains of *B. cinerea*. Primary applications were evaluated, with a multi-site inhibitor (chlorothalonil) or mixtures of non-cross resistant compounds (e.g. fludioxonil + cyprodinil) in alternation with other effective compounds with different modes of action (such as fenhexamid or boscalid). These treatments gave satisfactory disease control, despite the predominance of multi-fungicide resistant populations of *B. cinerea*.

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