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FIRST REPORT OF BOTRYTIS RESISTANCE IN RED BERRIES FRUIT TOWARDS FENHAXAMID AND FLUDIOXONIL + CYPRODINIL MIXTURE, AND ITS SENSITIVITY FEATURE TOWARDS OTHER SINGLE SITE FUNGICIDES IN MOROCCO

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A B S T R A C T

In this study single spores of seven *B. cinerea* isolates were collected from red-berries farms in the Loukkos and Gharb regions, North of Morocco, to evaluate their sensitivity to four commercial anti-botrytis fungicides registered in the country. All four fungicides contain different active ingredients namely: Iprodione, Fenhexamid, Thiophanate-methyl and mixture of Fludioxonil and Cyprodinil. Two main experiments were established *in vitro* for anti-*Botrytis* inhibitory assay; radial mycelia growth and spore germination inhibition tests at different concentrations. Fungicidal tests showed that all strains of *B. cinerea* were sensitivities to Iprodione with EC50 values (the effective concentrations to cause inhibition by 50%) ranging from 0.008 to 0.34 ppm inhibiting mycelia growth, and between 0.007 and 0.27 ppm inhibiting conidia germination. However, Fenhexamid did not show the same efficacy since all strains were resistant with EC50 values all higher than 5 ppm. For Thiophanate methyl, *Botrytis* strains responded differently to this product. Indeed, only 12.5% of strains were sensitive. EC50 values ranging from 83.28 to 2185.66 ppm for mycelia growth and from 73.17 to 3573.01 ppm for conidia germination. However, only 37.5% of the strains have shown sensitivity to Fludioxonil + Cyprodinil mixture with EC50 values ranging from 0.01 to 67.70 ppm for mycelial growth and between 0.001 and 5.81 ppm for conidia germination. These data may explain the non-efficacy of anti-Botrytis fungicides application in gray mold control in red berries fields in Morocco and highlights the need for new strategies for *Botrytis* management in different red-berries culture.

Keywords: *Botrytis cinerea, site specific fungicide, resistance, early monitoring*.

INTRODUCTION

Red fruits have been most commercially grown soft fruit because of their economic importance worldwide (Aprea *et al*. 2010). The Moroccan Ministry of Agriculture and Fisheries has reported 4162 hectares of red fruits production in the country with 150.000 tons, 97% was strawberries (MAFM 2013). Saying this, red fruits are highly susceptible to gray mold disease that causes devastating damages with important economic loss of such delicate cultures.

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Gray mold is the common disease caused by *Botrytis cinerea* fungus that attacks different host plants (about 200 species), including vegetables, ornamental, and fruits (Williamson *et al.* 2007). Unfortunately, there are still no resistant varieties developed against *Botrytis cinerea*. Therefore, gray mold control is based mainly on fungicide use (Leroux *et al.* 2002). Since 1970s', a diversity of single target site anti-botrytis fungicides were developed under different chemical groups holding diverse mode of actions (Leroux 2007). The chronological sequences of these fungicides development and use are cited as follows: benzimidazoles, dicarboximides, anilinopyrimidines, hydroxyanilides and phenylpyrroles (Williamson *et al.* 2007). However, chemical control seems to be not as effective as a solution for gray mold management since

sustainable culture yield is not satisfied and *Botrytis cinerea* still causes damages and reduces production, especially in greenhouse-grown cultures (Li *et al.* 2014 ; Panebianco *et al.* 2015). Different researches have revealed that the use of single anti-Botrytis fungicides lead to the emergence and spread of resistant *Botrytis* population (Bardas *et al.* 2010 ; Pokorny *et al.* 2016).

In Morocco, the first report of resistance to anti-*Botrytis* fungicides was established in a study conducted by Besri & Dietta (1985). The results of this study demonstrated that *Botrytis* strains developed a significant high resistance to Thiophanate methyl and Benomyl (Benzimidazol) but not Procymidone (dicarboximides). These findings have suggested the abusive use of benzimidazole products. In 2003, Hmouni *et al.* have studied sensitivity of six isolates of *Botrytis cinerea* collected from Gharb region to registered anti-botrytis fungicides. These isolates were resistant to Benomyl and Thiophanate methyl and even to dicarboximides reported as efficient before. These results show that the situation of Botrytis resistance to fungicides changes over time. This gives the possibility to detect new ineffective products against this fungus especially that the latter continues to cause serious damage in greenhouse production system of red fruits.

This study is intended to determine the level of *in vitro* sensitivities of *B. cinerea* isolates collected from different red berries farms in Morocco to Iprodione, Fenhexamid, Thiophanate methyl and Fludioxonil+Cyprodinil mixture. This information is very important to help with the development of strategies aiming to improve disease control and prevent the loss of fungicide efficiency.

MATERIALS AND METHODS

Obtaining isolates of *Botrytis:* Seven *B. cinerea* subcultures were isolated from different red berries including strawberries, raspberries, blueberries and mulberries collected from farms located in the region of Loukkos and Gharb in the North of Morocco. The isolate I68, Iprodione and Thiophanate-methyl multi-resistant strain collected from a strawberry field in Italy is also used in this experiment as a strain of reference. *Botrytis* isolate I68 belongs to the fungal collection of the Laboratory of Pesticides, Bio-pesticides and Environment, IAV Hassan II, Campus Rabat, Morocco.

Botrytis isolation from rotten red fruits was performed as described by Hmouni *et al.* (2003). To obtain *Botrytis* isolates, rotten red berries were incubated in a moist chamber for 2 to 3 days at room temperature to stimulate the pathogen sporulation. Hence, using a sterile needle and in sterile conditions, a small amount of spores mass and mycelium of *B. cinerea* was easily picked from the fruiting bodies developed on the top of rotten fruit and sub-cultured into a fresh Potatoes Dextrose Agar plates, (PDA Agar, difco). Many sub-culturing passages were completed to obtain pure culture of *Botrytis cinerea*. To obtain monospore cultures of *Botrytis*, all 7 to 10 days old *B. cinerea* subcultures growing on PDA medium were washed with a solution containing tween 20 at 2.5% and sterile distilled water. The suspension concentration was determined with hemocytometer. Afterwards, serial dilution was applied to the suspension and adjusted at 10^3 spores/ml then plated on slim agar layer at an amount of 0.5 milliliters (ml) and incubated for 24 to 48 hours (h) under white light. Germinated colonies were transferred onto a fresh PDA plate for two rounds to have the final *Botrytis* strains actually used in fungicides tests.

Fungicides used: Four fungicides were used for sensitivity test of *Botrytis* isolates, including Iprodione (Cabal 50 SC), Fenhexamid (Teldor 50 WG) and Thiophanate-methyl (Pelt 70 WDS) which belong to dicarboximides, hydroxyanilides and benzimidazoles chemical groups, respectively. Moreover, the product Switch 62.5 WG, a mixture formulation of Fludioxonil with Cyprodinil, which belong to anilopyrimidines and phenylpyrroles respectively, was also tested on *Botrytis* isolates. These four products containing five active ingredients belonging to different chemical groups, and hence, each holds a different biochemical mode of action from others. The concentrations used in sensitivity test were deduced from the literature based on recent research outcomes of preliminary tests for all four fungicides tested (Panebianco *et al.* 2015; Hmouni *et al.* 2003; Lopes *et al.* 2017). In fact, the concentrations range chosen has demonstrated somehow an effective inhibition of 50% of *Botrytis* isolates compared to the control and for every fungicide tested in previous works. Therefore, the concentrations used for Iprodione were 0 (control); 0.05; 0.1; 0.5; 1 and 10 parts per million. For Fenhexamid, the concentrations were 0; 1; 10; 100; 500; 1000 ppm. As for Thiophanate-methyl, the concentrations tested were 0; 250; 500; 1000; 2500; 5000 ppm and for Fludioxonil and Cyprodinil mixture, the concentrations were; 0; 0.01; 0.1; 0.5; 1; 10 ppm. *Botrytis* isolates sensitivity to these fungicides has been evaluated performing mycelium inhibition test on PDA

and spores germination test on water agar media.

Inhibition growth test: Mycelium growth test was performed as described by Hmouni *et al.* (1996). Autoclaved PDA media were cooled to about 45°C and amended with appropriate volumes of the fungicides to obtain the aforementioned concentrations. Then the media amended with fungicides were poured in 90 millimeter (mm) plates. 5 mm disc of the mycelium of 7 days old *Botrytis* was deposited in the center of plates, which were incubated at 25°C. After 4 days of incubation, mycelium growth was measured by calculating the average of two diameters of every *Botrytis* colony growing on PDA. The experiment was performed in three replicates for each fungicide concentration. The percentage of inhibition I (%) of *Botrytis* mycelium growth was calculated based on the equation 1.

$$I\ (\%) = \frac{A - B}{A} * 100 \quad \text{(equation 1)}$$

In this equation, A is average diameters of colonies in the control plates and B is average diameters of colonies in the plates amended with fungicide.

Conidia germination test: Conidial germination test was established following Leroux *et al.* (1992) Method. In fact, *B. cinerea* spores were collected by washing the surface of 10 days old colonies. The washing solution contained 2.5% Tween 20 and sterile distilled water. Afterwards, filtrate of spores' suspension was collected using muslin tissue, mixed with vortex for one minute then diluted. The spores' suspension was diluted to final concentration at 10^3 spores/ml with 0.20 ml, displayed on thin layer of water agar that is amended with different concentrations of fungicides. *Botrytis* spores germination was observed under optical microscope after 24 hours of incubation at 25°C. Only *Botrytis* spores with germ tube twice as long as their diameters have been considered in the measurement. Three replicates were performed for each fungicide concentration.

The percentage of inhibition of germination was calculated as described in equation 2 below.

$$\%\ \text{Inhibition of germination} = \frac{Total\ of\ spores\ observed - number\ of\ spores\ germinated}{Total\ of\ spores\ observed} \times 100 \quad (equation\ 2)$$

Data analysis: To identify sensitive and resistant isolates, reported data from previous work of discriminatory doses related to every fungicide have been applied. For instance, discriminatory doses of Iprodione were 2.5 ppm (Leroux *et al.* 1999). For Thiophanate methyl, most researchers agreed about 100 ppm as discriminatory concentration (Hsiang *et al.* 1991). For Fenhexamid, discriminatory concentration is 0.4 ppm (Leroux *et al.* 1999). For the commercial product « Switch » that contains two different active substances the lowest discriminatory concentration related to Fludioxonil was applied (0.2 ppm) (Leroux *et al.* 1999).

To determinate the concentrations of inhibition of 50% (IC50), the Probit analysis has been performed by log transformation of inhibition percentages and linear curve were drawn as Probit = f (Log_{10} concentration) extracted from the regression equation (y= ax +b). The IC50 of mycelia growth and spores germination were both determined and compared to the control. Statistical analyses have been established by variance analysis at 2 factors level (*Botrytis* strains/concentration) running SPSS. In the meantime, Tukey test has been performed to compare all means in order to cluster all *Botrytis* strains depending on their sensitivity or resistance towards every fungicide used in this experiment (P = 0.05).

RESULTS

Growth inhibition of *Botrytis cinerea:* All strains demonstrated by some means distinguishable sensitivity and/or resistance degrees to different fungicides tested comparing EC50 values of different *B. cinerea* strains as illustrated in table 1 below. Based on the discriminatory concentration, all strains were sensitive to Iprodione, including *Botrytis* isolate I 68. In fact, EC50 values of all strains ranged from 0.008 ppm to 0.34 ppm for Iprodione. On the other hand, all *B. cinerea* strains were resistant to Fenhexamid with EC50 values varied between 6.34 ppm and 1125 ppm. Strain F2 had no EC50 recorded since there was no inhibition of 50% of its mycelium even though fungicides concentrations were high.

EC50 values varied between 83.28 ppm and 2185.66 ppm in *Botrytis* strains to Thiophanate methyl. Only M1 had EC50 value less than 100 ppm (EC50 = 83.28 ppm) recorded, based on discriminatory concentration, and therefore, considered as a sensitive strain to

Thiophanate methyl. Yet, sensitivity degree of other strains was varied.

EC50 values varied between 0.01 ppm and 67.70 ppm for (Fludioxonil+Cyprodinil) mixture. In fact, F1, F2 and F3 demonstrated significant high sensitivity toward the product containing Fludioxonil and Cyprodinil with EC50 values 0.08 ppm, 0.02 ppm and 0.01 recorded respectively for sensitivity test. *Botrytis* strains M1, M2, M3, M4 and I 68 were resistant to Fludioxonil and Cyprodinil with EC50 values recorded 1.53, 1.36, 67.7, 0.32 and 0.22 ppm respectively and all significantly important than

Conidia germination of *Botrytis cinerea:* Sensitivity test of *Botrytis* strains to fungicides regarding their germination capacity gave almost as similar results as for mycelium growth inhibition with some exceptions as indicated in table 2 below. All *B. cinerea* strains were sensitive to Iprodione with significantly very low conidia germination percentage. Accordingly, EC50 varied between 0.007 ppm and 0.27 ppm. The EC50 values varied between 5.45 ppm and 3195.37 ppm in Fenhexamid sensitivity test. None of *B. cinerea* strains was sensitive to Fenhexamid due to the fact that all of them have exceeded the value of discriminatory concentration that is 0.4 microgram per milliliter (µg ml^{-1}).

Table 1: EC50 values of four fungicides tested for inhibition of mycelium growth of 8 strains of *B. cinerea*

	Iprodione		Fenhexamid		Thiophanate méthyle		Fludioxonil+ Cyprodinil	
Strains	EC50 (ppm)	Phenotype	EC50 (ppm)	Phenotype	EC50 (ppm)	Phenotype	EC50 (ppm)	Phenotype
F1	0.32 bcd	S	353.13 bc	R	1841.96 ab	R	0.08 d	S
F2	0.02 cd	S	-	R	1109.98 abc	R	0.02 d	S
F3	0.008 e	S	6.34 f	R	415.13 d	R	0.01 d	S
M1	0.13 d	S	19.62 e	R	83.28 e	S	1.53 b	R
M2	0.13 abc	S	21.22 e	R	474.74 cd	R	1.36 a	R
M3	0.34 a	S	1125.75 a	R	389.70 cd	R	67.70 ab	R
M4	0.13 ab	S	67.88 cd	R	2185.66 a	R	0.32 c	R
I 68	0.09 abc	S	82.53 d	R	1671.36 bc	R	0.22 c	R

[y] Numbers in each column followed by the same letter are not significantly different at P = 0.05 as determined by analysis of variance. Mean separation was conducted using Tukey's HSD test.

[z] S: sentitive; R: resistant.

Table 2: EC50 values of four fungicides tested for inhibition of conidia germination of 8 strains of *B. cinerea*

	Iprodione		Fenhexamid		Thiophanate Méthyle		Fludioxonil + Cyprodinil	
Strains	EC50 (ppm)	Phenotype	EC50 (ppm)	Phenotype	EC50 (ppm)	Phenotype	EC50 (ppm)	Phenotype
F1	0,27 a	S	3070 b	R	1535,20 b	R	0,02 e	S
F2	0,16 b	S	3195,37 a	R	3573,01 a	R	0,002 f	S
F3	0,007 e	S	5,45 f	R	1275,39 c	R	0,001 e	S
M1	0,019 de	S	16,00 e	R	73,17 g	S	1,17 b	R
M2	0,023 cd	S	9,24 f	R	257,80 f	R	0,77 c	R
M3	0,22 b	S	92,68 c	R	700,98 e	R	5,81 a	R
M4	0,004 de	S	30,00 d	R	2430,43 a	R	0,73 bc	R
I 68	0,07 c	S	64,30 c	R	1004,29 d	R	3,03 d	R

[y] Numbers in each column followed by the same letter are not significantly different at P = 0.05 as determined by analysis of variance. Mean separation was conducted using Tukey's HSD test. [z] S: sentitive; R: resistant

Consequently, all *B. cinerea* strains tested for Thiophanate methyl were considered resistant towards this fungicide except M1 strain, which is considered sensitive with EC50 = 73.17.

EC50 values of strains tested for Thiophanate methyl were the highest compared to other fungicides tested and varied between 73.17 ppm and 3573.01 ppm.

For Switch, the commercial product with (fludioxonil+cyprodinil) mixture, EC50 values of strains tested F1, F2 and F3 were 0.02, 0.002 and 0.001 respectively and estimated sensitive to Switch with their EC50 values significantly lower than discriminatory concentration that is 0.2 ppm. However, M1, M2, I68, M3 and M4 are considered resistant to Switch since their recorded EC50 concentrations were significantly higher than 0.2ppm that represents discriminatory concentration for this commercial product.

Multiple resistances to fungicide

Multi-fungicide resistance has been observed towards Thiophanate methyl, Fenhexamid and (Fludioxonil+Cyprodinil) mixture in *B. cinerea* population that is tested in the present study. In fact, 87.5% of *B. cinerea* strains were resistant to Thiophanate-methyl and to Fenhexamid, 50% was resistant to Thiophanate-methyl and to (Fludioxonil+Cyprodinil), 62.5% was resistant to Fenhexamid and to (Fludioxonil+Cyprodinil). Finally, 50% of these strains showed multiple resistant towards three fungicides altogether.

DISCUSSION

Botrytis cinerea is responsible for some of the most serious problem of fungicide resistance in the world (Leroux *et al.* 2002; Fernández-Ortuño *et al.* 2013; Lopez *et al.* 2017). In this study, many strains were resistant to the majority of the tested fungicides. This indicates the serious problem of resistance in the regions under study. Fenhexamid was not able to inhibit mycelium growth of any of *B. cinerea* strain even though at 1000 ppm. This may be due to the excessive use of the product (Fenhexamid) by Moroccan farmers. Similar studies in the United States have also demonstrated *Botrytis* resistance to Fenhexamid (Grabke 2014; Cosseboom 2018; Amiri and Peres 2014). Similar results were found in researches established by Yin *et al.* (2014) in China.

The presence of resistant strains may not be due only to the excessive use of the product, for in our case the F3 strain was collected from a farm that is only three years old. Despite this fact, the strain showed its resistance to the product, which gives the hypothesis that there are strains that are naturally resistant to Fenhexamid. In the same vein, Adamo (2016) has observed that there were fields with no history of use of Fenhexamid and yet resistance of *Botrytis* has been reported, whereas in other fields where Fenhexamid is well applied, no *Botrytis* resistance was spotted. Walker *et al.* (2011) have worked on *Botrytis Pseudo-cinerea,* a species closely related to *B. cinerea*, and have shown *Botrytis Pseudo-cinerea's* natural resistance towards Fenhexamid. On the contrary, Panebianco *et al.* (2015) have demonstrated significantly high sensitivity to Fenhexamid by *Botrytis* population in Italy. In this study, no isolates resistant to Fenhexamid were found and the frequency distributions of their EC50 values were ranged from 0.0002 to 0.09 mg mL^{-1}. Benzimidazole fungicides have been widely used in agriculture since 1970s (Walker et al. 2011), and resistance to this class of fungicides in *Botrytis cinerea* is reported worldwide (Zhang *et al.* 2009; Fernández-Ortuño *et al.*2014). In fact, different benzimidazoles resistant phenotypes are stable whatever fungicide is applied (Yourman *et al.* 2001). Thus, *Botrytis* resistance to this class of fungicide was expected in our experiment. Consequently, this fungicide was not able to inhibit neither mycelium growth nor conidia germination of *Botrytis* strains. Similar results have been reported by Hmouni *et al. (2003)* in Morocco, Lopez *et al.* (2017) and Baggio *et al.* (2018) in Brazil, Cosseboom (2018) and Avenot et al. (2019) in USA and by Fan *et al.* (2016 ; 2017 ; 2019) in China.

The population of *B. cinerea*, the concern of this paper, has been proven to be sensitive towards Iprodione when testing their mycelium growth and conidia germination potential. These results are in agreement with previous studies. In fact, research conducted by Pokorny *et al.* (2016) and Fernández-Ortuño *et al.* (2013) reported the effective sensitivity towards Iprodione in the *B. cinerea* population examined. For instance, 74.3% of *Botrytis* strains were sensitive to Iprodione in Lopes *et al.* (2017) study.

Similar results were reported by Grabke (2014). Grabke's study revealed that only 2% of the population showed moderate resistance and 17.6% showed low resistance to Iprodione. By the same token, recent studies have demonstrated that strains of *B. cinerea* were sensitive to Iprodione and that may be because Iprodione resistance is reversible. Cosseboom (2018) has found that only 14% of *B. cinerea* strains were resistant to Iprodione with significant differences in resistance frequency ($p < 0.0001$) recorded early in the season (0 to 8 fungicide applications) and at the end of

season (16 to 26 fungicide applications). In Sicily, Italy, Panebianco *et al.* (2015) have found that only 10.3 % of *Botrytis* isolates were resistant to Iprodione.

On the other side of the coin, the findings of other studies have found the opposite of the results discussed above. First cases of resistance reported in the chemical group Dicarboximides were in 1979 and were related to Iprodione (Eckert 1988). Previous research established by Hmouni *et al.* (2003; 1996) has revealed resistance to Iprodione in *B. cinerea* population studied in Morocco and Tunisia. . In a recent study done on *B. cinerea* population of California, Avenot *et al.* (2018) have found that 62% of *B. cinerea* showed low resistance to Iprodione (1 <EC50 <10µg / mL) and only 38% of strains were sensitive to the product. Similar results were reported on *B. cinerea* population studied in Brazil conducted by Baggio *et al.* (2018), the study Showed that the Disease incidence of iprodione-treated fruit inoculated with resistant isolates ranged from 60.2 to 83.2%.

In this study, *Botrytis* strains showed distinctive responses on sensitivity towards Fludioxonil + Cyprodinil mixture. In fact, *Botrytis* strains collected from Loukkos region demonstrated resistance to this mixture. However, *Botrytis* strains provided from Gharb region were sensitive to the same product. At this level, it may be inferred that *Botrytis* population resistance to the mixture product is highly related to the excessive application of this fungicide in Loukkos region. It is worth mentioning that Fludioxonil and Cyprodinil resistance is not really common in *B. cinerea (*Panebianco *et al.* 2015; Fernández-Ortuño *et al.* 2013; Avenot *et al.* 2018). Fludioxonil and Cyprodinil are highly appreciated as active substances against *B. cinerea* efficacy in gray mold control (Kim *et al.* 2007; Kim *et al.* 2016; Liu *et al.* 2016).

Future research should focus on early monitoring resistance level of *Botrytis cinerea* towards different fungicides in different farming systems in Morocco. Moreover, further *in vitro* and *in vivo* tests for all registered anti-Botrytis products to evaluate their potential to control *Botrytis* strains are highly recommended. Integrated management program based on early diagnosis and prophylactic measure of *Botrytis cinerea* resistance in red fruit fields in the country should be developed and implemented.

CONCLUSION

B. cinerea sensitivity tests to different fungicides revealed a wide variability response level of sensitivity in *B. cinerea* strains. Efficacy of Iprodione has been well demonstrated among other fungicides tested during this experiment, whereas Fenhexamid and Thiophanate methyl were less efficacious in *Botrytis* strains control *in vitro*. Phenotype stability of *Botrytis* strains regarding the geographical regions under study and their sensitivity towards the same fungicide that we applied have been revealed when using Fludioxonil + Cyprodinil mixture. In fact, *Botrytis* strains showed different sensitivity responses towards Fludioxonil + Cyprodinil mixture and that may be explained by the fact of extensive and sometimes abusive use in some regions compared to others. Hence, all information generated in this study is of paramount importance to the elaboration of strategies regarding monitoring, and therefore, the best management of gray mold is in designing efficient fungicide application for red berries cultures in Morocco.

Significance of statement: A major current focus in red berries production system management in worldwide is early monitoring of fungicide resistance development. This study first report resistance case of *Botrytis cinerea* to Fenhexamid and (Fludioxonil + Cyprodinil) mixture in Morocco and its sensitivity to other anti-Botrytis fungicides. Detection of resistant *Botrytis* isolates collected from conventional red berries fields point out an important early diagnosis to be investigated in early monitoring of *Botrytis* resistance in red berries production system. That is, this study will help researchers as much as agricultural and phytosanitary industries to establish and resolve *Botrytis cinerea* management in such delicate cultures.

Conflict of interest: The authors have declared that there is no conflict of interest

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Contribution of Authors:
All authors contributed equally to this research.