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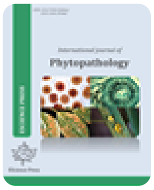

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FLUDIOXONIL, FENHEXAMID AND BOSCALID-INSENSITIVE *BOTRYTIS FABAE* ISOLATES COLLECTED FROM MOROCCAN FABA BEAN FIELDS

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A B S T R A C T

Chocolate spot, caused by *Botrytis fabae*, poses a significant threat to faba bean production in Morocco, leading to decreased crop quality and quantity. This study assesses the sensitivity of *B. fabae* isolates to three fungicides-fenhexamid, fludioxonil, and boscalid-commonly used in Moroccan faba bean cultivation. Fungicide sensitivity tests were conducted on 22 single-spore isolates of *B. fabae* from five faba bean-growing regions in Morocco. The Growth Inhibition Percentage (GIP) of each isolate was calculated using an automated quantitative (AQ) test to determine sensitivity profiles to the three fungicides. Six representative isolates from different sensitivity profiles were tested against a range of concentrations of fenhexamid, fludioxonil, and boscalid (0–1,000 mg/L) to determine EC50 values. *In vivo* greenhouse experiment evaluated the efficacy of two fungicides fludioxonil and boscalid against *B. fabae* isolates with distinct sensitivity profiles. Mass Disease Index (MDI) was used to evaluate fungicide effectiveness. Results indicated that 45% of the isolates demonstrated resistance to fludioxonil, and 14% exhibited resistance to boscalid. Two of the three boscalid-resistant isolates were also resistant to fludioxonil. In contrast, the majority (95%) of isolates were moderately resistant or sensitive to fenhexamid. The calculated EC50 values of fludioxonil, boscalid and fenhexamid against *B. fabae* isolates clearly distinguished sensitive from resistant isolates; for resistant isolates EC50 were ≥ 140 mg/L while the values for sensitive isolates were ≤ 30 mg/L. *In vivo* trials revealed inefficacy of fludioxonil and boscalid against resistant isolates. These findings highlight the emergence of fludioxonil and boscalid insensitivity in *B. fabae* in Morocco, indicating the need for continuous monitoring of fungicide resistance in chocolate spot management. Future research should develop methods to manage fungicide resistance, aiming for sustainable control of chocolate spot in faba bean.

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INTRODUCTION

Chocolate spot (CS), caused by *Botrytis fabae*, is the main fungal disease and yield-limiting factor in Moroccan faba bean (*Vicia faba)* production (Bouhassan *et al.*, 2004; Krimi Bencheqroun, 2024). The disease leads to annual yield loss costs of A$1.4 million in Australia (Murray and

Brennan, 2012). A recent study in Ethiopia by Tiki *et al.* (2020) reported relative yield losses of 28.5–37.3%, emphasizing the need for continuous monitoring and management to sustain faba bean productivity both in Morocco and worldwide. Disease management mainly depends on the foliar application of chemical treatments, especially site-specific fungicides. However, *Botrytis* spp. is considered a high-risk pathogen for developing resistance to site-specific fungicides (Richards *et al.*, 2021). The high genetic diversity of *Botrytis spp.* enhance its rapid environmental adaptability and the potential to develop resistance to commonly applied fungicides (FRAC, 2019). A main example is *Botrytis cinerea*, which has demonstrated resistance across diverse crops, including strawberries, vineyards, and tomatoes (Cosseboom *et al.*, 2019). Therefore, continuous fungicide resistance monitoring is essential for preventing or delaying the breakdown of fungicide efficacy (Liu *et al.*, 2021). However, despite the critical need for understanding and managing fungicide resistance, there's a lake of informations about *B. fabae* sensitivity at both the national and international levels. To date, no fungicide resistance or failure to control chocolate spot has been reported in Moroccan faba bean fields. This study aimed to investigate the sensitivity of *B. fabae* isolates to three commonly used active substances, including fenhexamid, fludioxonil and boscalid. The assessments were carried out in both *in vitro* and *in vivo* under greenhouse conditions to evaluate the effectiveness of these fungicides to control the disease.

MATERIAL AND METHODS

Fungal Isolation

Infested faba bean samples were collected from five faba bean-growing regions in Morocco (Abda, Doukkala, Saiss-Taza, Zemmour - Zaër, et Chaouia) during the 2016 cropping season. Leaf samples were cut into 1 cm pieces, surface-sterilized with 5% sodium hypochlorite solution for one minute, washed twice with sterile distilled water, and plated on potato dextrose agar (PDA). Single-spore of 22 isolates were then purified and incubated at 22 ± 2 °C with a 12-hour light-dark cycle for 10 days. The isolates species were characterized morphologically using identification keys (Mirzaei *et al.*, 2008; Hennebert, 1973).

Fungicide Sensitivity Test

All *B. fabae* isolates were evaluated for their sensitivity to fungicides by estimating the fungal biomass using an automated quantitative test (AQ) according to Campia *et al.* (2017). Three commonly used botryticide substances, technical grade (Sigma Aldrich) were evaluated for their ability to inhibit fungal growth of, including fenhexamid (a keto-reductase inhibitor), fludioxonil (a phenylpyrrole), and boscalid (a succinate dehydrogenase inhibitor).

The discriminatory doses for fenhexamid (0.5 mg/l), fludioxonil (0.1 mg/l) and boscalid (1.2 mg/l) were adapted from Toffolatti *et al.* (2020). The active substances were dissolved in dimethyl sulfoxide (DMSO) for consistent solubility . The conidial suspension of each isolates were adjusted to 2x10^4 conidia/mL.

The sensitivity test was carried out in 96-well microtiter plates (Sero-wel; Bibby Sterilin Ltd) using minimal cultural medium (MCM). 50 µL of conidial suspensions and 50 µL of medium were added in each well. Two replicates were used for each concentration of the active substances. Growth inhibition percentage (GIP) was calculated by measuring the absorbance of each well at 492 nm before and after the incubation period of 72h at 20°C using the following formula (Vercesi *et al.*, 2014);

$$GIP\% = [(\mathrm{ACtf} - \mathrm{ACt_0}) - (\mathrm{AFt\,f} - \mathrm{AFt_0})] \times 100 / (\mathrm{ACt\,f} - \mathrm{ACt_0})$$

Where: $\mathrm{ACt_0}$ and $\mathrm{AFt_0}$ are the initial absorbances of control (C) and fungicide-treated (F) samples, and ACt f and AFt f are the final absorbances. Isolates were classified as resistant if the GIP was less than 5% in medium amended with discriminatory doses, moderately resistant if it was between ≥5-50%, and sensitive when GIP was higher than 50% (Vercesi *et al.*, 2014).

Fungicide EC50 Value

The EC50 represent the concentration of fungicide needed to reduce mycelial growth by 50%. Six representative isolates from different sensitivity profiles (resistant and sensitive) for fenhexamid, fludioxonil, and boscalid were tested against a range of fungicide rates to deter-mine EC50 values. Fungal plots of -day-old pathogen mycelium were placed on PDA plates (Potato Dextrose Agar) amended with six different concentrations of fungicides : 0, 25, 50, 250, 500, 1000 mg/l. The experiment was conducted in triplicate for each fungicide concentration. The percentage of inhibition of pathogen mycelial growth (I%) was calculated using the formula (Taskeen-Un-Nisa *et al.*, 2011);

$$I\ (\%) = \frac{A - B}{A} * 100$$

Where, A: is the mean diameter of colonies in control plates, B: is the mean diameter of colonies in plates amended with fungicide.

Percentage of mycelial growth inhibitions for tested fungicides were analyzed using probit analysis to determine the EC50 values.

Fungicides Control Efficacy *In Vivo*

The ability of fludioxonil and boscalid to control *B. fabae* resistant isolates was evaluated *in vivo* in the greenhouse. Fenhexamid was not selected to be tested in this experiment since only one isolate was found resistant in the *in vitro* fungicide sensitivity test. Two isolates with different sensitivity profiles were selected for this test; one isolate with dual resistance to fludioxonil and boscalid, and another isolate sensitive to both substances.The seeds of the sensitive faba bean cultivar ELISAR were surface disinfected and planted in 14 cm diameter pots filled with a mixture of sterilised soil, peat, and sand (3:1:1, v:v:v). Four plants per pot were grown under controlled conditions with a temperature of 20/16°C and a photoperiod (14/10 h day/night) during three weeks (Bouhassan *et al.*, 2004). Faba bean seedlings were treated individually at the 4-leaf stage by spraying with either water (control) or 60 mg a.i./l of fungicide Switch 62.5 WG (fludioxonil 25%) or 125 mg a.i./l of Signum WG (boscalid 27 %) to runoff using a hand sprayer. After 24 hours, seedlings were inoculated by spraying 2 ml/plant of conidial suspension (10^5 spore /ml) of *B. fabae* isolates (Bouhassan *et al.*, 2004). Greenhouse trial was conducted in a randomized complete block design with three replications for each treatment. Foliar disease severity was rated every two days after inoculation based on a scale of 0 to 9 (Ding *et al.*, 1993). The disease severity scores were used to calculate the Mass Disease Index (MDI) after 16 days using the formula (Hanounik and Hasanain, 1986):

$$MDI\ \% = \frac{\sum(\mathrm{N} * \mathrm{V})}{9\ \mathrm{N}} * 100$$

Where, N= number of infected leaves, V= numerical score, 9 = highest grade in the category.

Data Analysis

All data were subjected to analysis of variance. One-way ANOVA was used to evaluate growth inhibition percentage (GIP), while two-way ANOVA was applied to examine interactions between profiles and fungicides.

Post-hoc tests, Tukey's HSD, were conducted to determine mean differences between treatments. The significance level adopted for all statistical analyses was set at 0.05. Statistical analyses were performed using SPSS software (IBM SPSS Statistics 22).

RESULTS

Fungicides Sensitivity

Based on the growth inhibition percentage (GIP), the results showed varying levels of resistance and sensitivity to the tested fungicides among the *B. fabae* isolates. For fludioxonil, the majority of isolates were either resistant (10 isolates) or moderately resistant (9 isolates), with only three isolates exhibiting sensitivity to the fungicide. For boscalid, most isolates were either moderately resistant (17 isolates) or resistant (3 isolates), while only two isolates were sensitive.Two of the three boscalid-resistant isolates were also resistant to fludioxonil. Regarding fenhexamid, only one isolate was resistant, whereas the remaining isolates were either moderately resistant (16 isolates) or sensitive (5 isolates). This resistant-fenhexamid isolate was sensitive to both fludioxonil and boscalid (Figure 1).

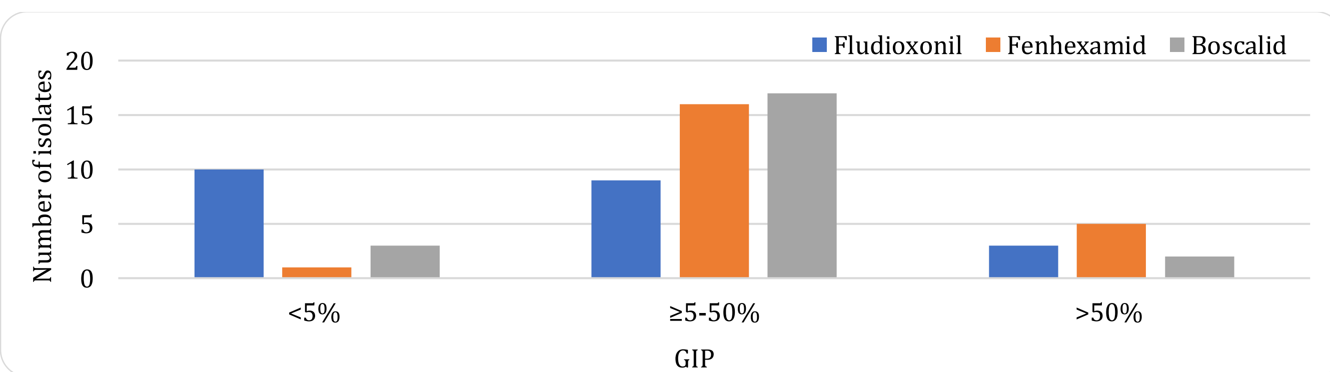

Figure 1. Distribution of GIP (The Growth Inhibition Percentage) values for *B. fabae* isolates in response to fludioxonil, fenhexamid and boscalid.

Analysis of the frequency profiles of the *B. fabae* isolates evaluated in this study showed that isolates from all five regions of Morocco exhibited resistance to fludioxonil. In contrast, resistance to boscalid was observed in isolates from three of the five regions (Zemmour-Zaër, Saiss-Taza, and Chaouia). Resistance to fenhexamid was only detected in the Doukkala region. All isolates from the Abda region showed either resistance or moderate resistance to the all tested fungicides (Table 1, Figure 1).

Table 1. Classification of fungicide sensitivity of *B. fabae* isolates collected from faba bean fields in five regions in Morocco.

Regions	Number of isolates	Frequency of profiles by region (%)								
		Fludioxonil			Fenhexamid			Boscalid		
		R	MR	S	R	MR	S	R	MR	S
ZZ	5	40	40	20	0	40	60	20	60	20
ST	8	37,5	62,5	0	0	100	0	12,5	75	12,5
DK	4	50	25	25	25	75	0	0	100	0
CH	3	33,3	33,3	33,3	0,0	33,3	66,7	33,3	66,7	0,0
AB	2	100	0	0	0	100	0	0	100	0

ZZ: Zemmour - Zaër ST: Saiss-Taza, DK: Doukkala, CH: Chaouia, AB: Abda, R: Resistant,MR: Moderately resistant, S: Sensitive.

Fungicide EC50 Value

The calculated EC50 values of fludioxonil, boscalid and fenhexamid against *B. fabae* isolates clearly distinguished sensitive from resistant isolates. The EC50 values of the sensitive *B. fabae* isolates to fludioxonil, boscalid and fenhexamid were 29.5mg/l, 29mg/l and 18mg/l respectively, whereas the EC50 values of the resistant isolates to these fungicides were respectively 188.5mg/l, 140mg/l and >250mg/l (Table 2). A high EC50 value for fenhexamid indicates a requirement for higher concentrations to achieve a 50% inhibition response, signifying a low sensitivity of this isolate to the treatment.

Fungicides Control Efficacy *In Vivo*

The efficacy of fludioxonil and boscalid in controlling the chocolate spot disease on plants inoculated with resistant *B. fabae* isolate (FluR/BosR) in comparaison to sensitive isolate (FluS/BosS) (Figure 2)was evaluated *in vivo* in the greenhouse. A significant effect of treatment and isolate interaction was observed at *P<0.05* F(3, 30) = [9.27], lesions developed on treated plants inoculated with fludioxonil and boscalid-resistant isolates (MDI= 44% and 41% for fludioxonil and boscalid treatments respectively) were statistically similar to non-treated plants (MDI=47%). Whereas the disease caused by sensitive isolates was significantly controlled with these treatments compared to the control (MDI=33 % and 37% for fludioxonil and boscalid treatments respectively) (Figure 2).

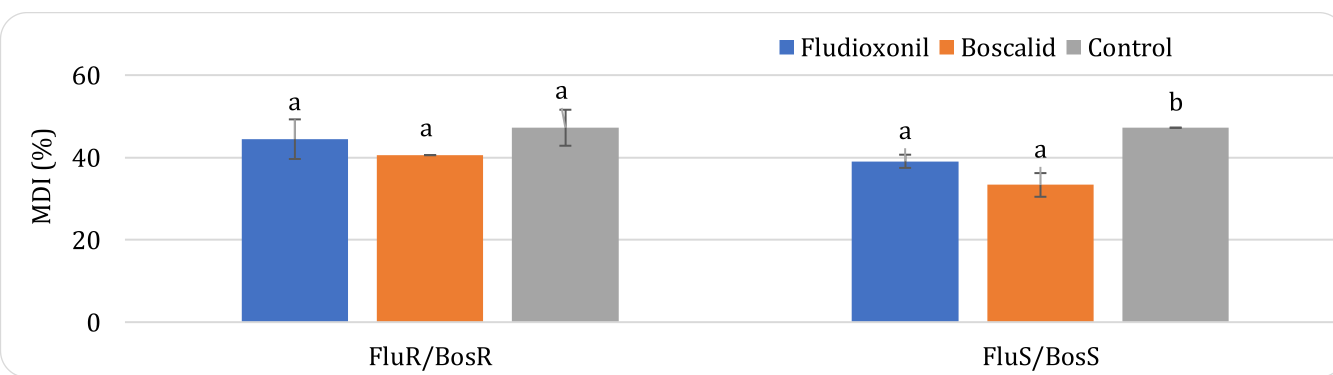

Figure 2. Effect of fludioxonil and boscalid treatments on the mean disease index (MDI) of plants inoculated with resistant (FluR/BosR) and sensitive (FluS/BosS) isolates of B. fabae.

Different lower-case letters indicate a significant difference (P<0.05) when analysed by Duncan's multiple range test. The value is the mean ± SE.

Table 2. EC50 value of fludioxonil, boscalid and fenhexamid against B. fabae isolates with different levels of fungicide resistance.

	Fludioxonil		Boscalid		Fenhexamid	
Sensitivity[1]	Resistant	Sensitive	Resistant	Sensitive	Resistant	Sensitive
EC50 (mg/l)[2]	188,5	29,5	140	29	> 250	18

[1] The sensitivity of isolates to fungicides was determined according to AQ test with sensitive isolates having GIP>50% and resistant isolates having GIP<5% in medium amended with discriminatory doses

[2] EC50 value: Fungicide concentration that results in 50% inhibition of mycelial growth relative to control

DISCUSSION

In this study, we evaluated the resistance of *B. fabae* isolates of faba bean to the three single-site fungicides including fludioxonil, fenhexamid, and boscalid widely used in Morocco. Our *in vitro* fungicide sensitivity assay using the QA test revealed a spectrum of resistance profiles among *B. fabae* isolates. While some isolates demonstrated sensitivity (S) to fludioxonil, boscalid, and fenhexamid, others exhibited varying degrees of resistance (R).

Our findings suggest that the AQ assay could be a valuable tool for fungicide sensitivity testing in *B. fabae* populations. This aligns with previous research by Raposo *et al.* (1995) and Tremblay *et al.* (2003) who demonstrated the effectiveness of the AQ assay for *B. cinerea* and *B. squamosa*, respectively. Furthermore, Campia et *al.* (2017) successfully utilized the AQ test to assess *B. cinerea* sensitivity to common fungicides in northern Italy, indicating the assay's potential broad applicability across diverse fungal species and geographical regions.

The evaluation of EC50 of fludioxonil, boscalid and fenhexamid against *B. fabae* isolates with different levels of fungicide resistance , showed a link between EC50 value and the profiles obtained from the AQ test. Isolates classified as resistant (R) based on the AQ test exhibited higher EC50 values compared to sensitive (S) isolates. This finding suggests that the AQ test can effectively predicts the resistance of *B. fabae* isolates to various fungicides. The results are consistent with Cosseboom and Hu (2021) study, confirming the synegry between the AQ test results and EC50 values in characterizing fungicide resistance in *B. cinerea.*

The *in vivo* assessment, in greenhouse conditions, of fludioxonil and boscalid effect in controlling the chocolate spot revealed a lose of efficacy on faba bean seedling inoculated with resistant isolates. These results confirm the distinction between resistant (R) and sensitive (S) isolate profiles as determined by the AQ test. For instance, Raposo *et al.* (1995) observed strong agreement between the AQ assay and *in vivo* fungicide efficacy in *B. cinerea*. Similarly, Wu *et al.* (2014) reported a close correlation between *in vitro* and *in vivo* evaluations of fungicide sensitivity, supporting the broader applicability of the AQ assay.

Fludioxonil and boscalid-Resistant isolates were prevalent across the surveyed regions, with 45% and 14% of isolates, respectively. The widespread resistance to fludioxonil across all regions suggests the impact of its broad application on various crops and diseases. Conversely, resistance to boscalid was observed in three regions, likely due to its more targeted use for the control of *Botrytis* species in specific regions (ONSSA, 2024). Interestingly, only one isolate was found resistant to fenhexamid in our studied population. This result suggest a lower risk of fenhexamid-resistant development compared to the two other fungicides. A previous research, also, observed a high level of *B. cinerea* susceptibility to fenhexamid, with only 10% of isolates exhibiting resistance (Avenot *et al.*, 2020). Other study indicates that the absence of fenhexamid-resistant isolates of *B. cinerea* in Sicilian vineyards, despite its widespread use, can be attributed to fenhexamid's classification as a low-risk fungicide for resistance development (Brent and Hollomon, 1998; FRAC Code List, 2015). However, a high potential of *B. cinerea* to develop resistance to boscalid was observed by several studies (Bardas *et al.*, 2010; Fernández-Ortuño *et al.*, 2012; Walker, 2013; Yin *et al.*, 2011). Similarly, the emergence of fludioxonil resistance in *B. cinerea* isolates has been reported several countries; in United States (Fernández-Ortuño *et al.*, 2014), Germany (Rupp *et al.*, 2017), and China (Sang *et al.*, 2018).

The emergence of fludioxonil and boscalid-resistant *B. fabae* isolates can be du to a high genetic diversity of this pathogen in the Moroccan population. Pathogen populations with a high evolutionary potential are more likely to overcome fungicide effectiveness than pathogen populations with low evolutionary potential (McDonald and Linde, 2002). In deed, in a recent study, population structure analysis of Moroccan population of *Botrytis* spp. on faba bean revealed a high gene flow among regions, suggesting a high evolutionary potential and a spread of novel alleles that might breakdown fungicide efficacy (Aouzal *et al.*, 2022). Further researchs are required to pinpoint the specific mechanisms driving fungicide resistance. The developpement of resistance to multiple fungicides, including fenhexamid and boscalid, in *B. fabae* isolates can pose a significant challenges for disease management.

This report constitutes the first documentation of insensitivity of *B. fabae* on faba beans to fludioxonil and boscalid. Among *Botrytis* species, resistance to the fungicides fludioxonil and boscalid has only been reported in isolates of *B. cinerea* (Cosseboom *et al.*, 2019; Liu *et al.*, 2021). To determine whether the resistance reported in this study affects the performance of boscalid and fludioxonil in the field, it will be important to assess the ability of resistant isolates to cause chocolate spot disease in bean fields treated regularly with these fungicides. The identification of *B. fabae* resistant isolates on faba bean to these active substances widely used against *B. fabae* highlights the importance of resistance management strategies.

Effectively managing fungicide resistance requires a multifaceted approach that prioritizes prevention. The basis of this strategy is fungicides rotation, by switching between fungicides with different modes of action when controling fungal diseases (Sautua *et al.*, 2019; Habib *et al.*, 2020; Müller *et al.*, 2021). Early detection of fungicide resistance can, also, allow a timely adjustments of fungicide programs, ensuring their continued effectiveness (Weber and Hahn, 2011). Furthermore, incorporating resistant cultivars, specifically developed through breeding programs to possess inherent resistance to the target fungus, can minimize reliance on fungicides altogether. This reduces selection pressure for resistant fungi, preserving the effectiveness of fungicides for other diseases (Brent and Hollomon, 1998). Therefore, by implementing a comprehensive strategy that incorporates these elements, we can significantly slow the emergence and spread of fungicide-resistant fungal populations.

CONCLUSIONS

This study highlights the emergence of fludioxonil and boscalid insensitivity in *B. fabae* in Moroccan faba bean fields, underlining the importance of monitoring fungicide effectiveness regularly. The identification of resistant isolates underscores the need for proactive resistance management, incorporating diverse strategies such as early detection and integrating alternative disease control methods. Addressing fungicide insensitivity through tailored interventions will be essential for maintaining the sustainability of faba bean production in Morocco. Further studies should explore the underlying mechanisms driving resistance and evaluate alternative control measures to mitigate the spread of insensitive isolates.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, S.K.B., and S.A.; methodology, S.K.B., S.A., and H.H.; software and formal analysis, S.A., S.K.B., and L.Z; investigation.; writing—original draft preparation, S.A. and S.K.B.; writing—review and editing, S.A, S.K.B., H.H and R.M; supervision, S.K.B., and H.H.; project administration S.K.B. All authors have read and agreed to the published version of the manuscript.

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