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Sensitivity to fungicides of *Botrytis cinerea* (Pers.) isolated from raspberry (*Rubus idaeus* L.)

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ABSTRACT

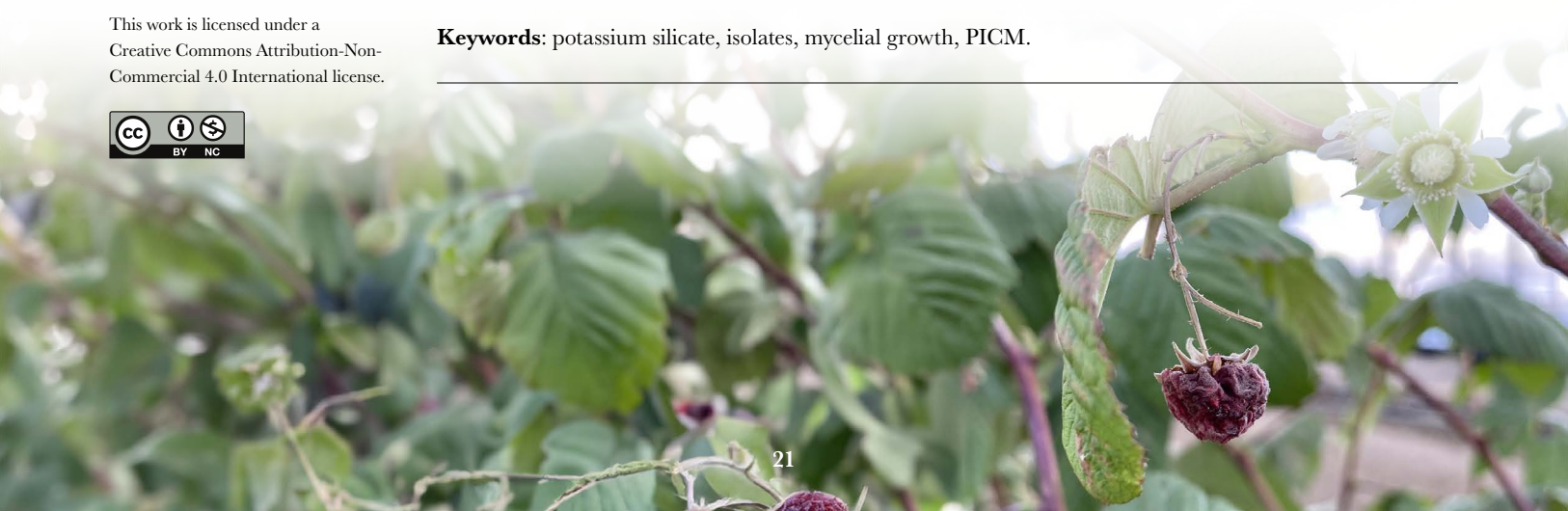
Objective: To evaluate the sensitivity of *B. cinerea* isolated from raspberry to nine fungicides alone and in combination with potassium silicate.

Design/methodology/approach: The study evaluates the sensitivity of four isolates obtained from four raspberry plantations in October 2022, which were identified in a previous study as *B. cinerea* based on morphological, morphometric and molecular characteristics, to nine fungicides alone and combined with potassium silicate. *B. cinerea* was planted in PDA culture medium modified with fungicides plus potassium silicate, and mycelial growth and mycelial growth inhibition percentage (PICM) were evaluated. A completely randomized design with six repetitions and two controls was used, an ANOVA and Tukey's mean comparison test were performed.

Results: *B. cinerea* isolated from CITAP showed lower growth with fluazinam (PICM=100), while with azoxystrobin it presented a PICM equal to 0. *B. cinerea* isolated from Paso del Cristo and Sierra Negra 2 had lower growth with fluazinam (PICM=100), while with boscalid and azoxystrobin it showed a PICM equal to 0. In Sierra Negra 1, iprodione controlled *B. cinerea* better with a PICM equal to 100, while azoxystrobin showed a PICM equal to 0. All the isolates were sensitive when fungicides were combined with potassium silicate.

Findings/conclusions: All the isolates were sensitive to the fungicides fluazinam, fenhexamid, thiophanate methyl, captan, pyrimethanil, fludioxonil and iprodione. The isolates from Sierra Negra 1 and CITAP were sensitive to boscalid, while those from Sierra Negra 2 and Paso del Cristo were insensitive; 100% of the isolates were insensitive to azoxystrobin, which suggests that they could be resistant; finally, potassium silicate potentiates the effect of fungicides.

Keywords: potassium silicate, isolates, mycelial growth, PICM.



INTRODUCTION

Raspberry (*Rubus idaeus*) cultivation is produced in different parts of the world and approximately 886 538.5 t are harvested, where the main producer is the Russian Federation, followed by Mexico with a production of 165 677 t (FAOSTAT, 2023); the main producing states are Jalisco, Michoacán, Baja California, Guanajuato and Puebla (SIAP, 2023); the most important disease in pre- and post-harvest of this crop is gray mold induced by *Botrytis cinerea*, where the most severe damage is in the fruit (Fillinger and Elad, 2016), the symptoms are brown soft rotting, and conidiophores and conidia are formed which together form the gray mold and then the infructescence dries up and mummifies (Carisse *et al.*, 2015). If the crop is not protected with fungicides of different modes of action, the fungus could generate resistance in addition to destroying the crop in a matter of weeks (Fillinger and Elad, 2016; Nieto *et al.*, 2022). Chemical control is the main efficient means against gray mold, and currently there are seven modes of action of single-site fungicides in addition to the multi-site fungicides (Fillinger and Walker, 2016); the application of these can increase the production costs and generate selection of resistant populations (Fernández-Ortuño, 2014). One alternative to counteract this is the use of alternative products which, when combining with different fungicides, are efficient in potentiating their effect; this is the use of Si (Nieto *et al.*, 2022), which is the natural form in which plants accumulate it in intercellular spaces, the cell wall, the cell lumen, epidermis and cuticle, and which possibly acts as an adverse factor in the adequate nutrition of fungi (Datnoff *et al.*, 2011). Jennings (2007) mentions that when the Si adheres to the cell wall, the enzymes from the hyphae do not act efficiently in unfolding the cellulose to glucose, and the fungi nutrition is affected negatively so the disease decreases; therefore, Si reduces the impact and severity of diseases of the plants caused by biotrophic, hemibiotrophic and necrotrophic pathogens (Lopes *et al.*, 2014; Rodrigues *et al.*, 2015, Nieto *et al.*, 2022), in addition to activating the natural defenses of plants, primarily the expression of resistance genes (Fauteux *et al.*, 2005). Different *in vitro* studies of chemical control of *B. cinerea* have been conducted where the action mechanism of fungicides are known exactly, but information of *in vitro* control with potassium silicate is limited; it is believed that Si is toxic to it, and in its presence the fungus cannot absorb the sources of carbon from the culture medium reducing its hyphae growth rate (Velazquez *et al.*, 2016). Because of the aforementioned, the objective of this study was to evaluate the sensitivity of *B. cinerea* isolated from four commercial crops of raspberry in the state of Puebla, to nine fungicides of different chemical families, alone and in combination with potassium silicate.

MATERIALS AND METHODS

Origin of the isolates

Four isolates were used selected from 40 obtained from four raspberry plantations were used: Centro de Innovación Tecnológica en Agricultura Protegida (CITAP) in the Universidad Popular Autónoma del Estado de Puebla (UPAEP) (18.930912, -98.398468), Paso del Cristo (18.885798, -98.443146), Sierra Negra 1 (18.503310, -97.464932), and Sierra Negra 2 (18.8754977, -98.4087937). They were characterized by a previous

study and correspond to *Botrytis cinerea* GenBank (ITS: OQ618427; G3PDH: OQ630985; HSP60: OQ630986; RPB2: OQ630987).

Sensitivity to fungicides and potentiation with potassium silicate

The sensitivity of four isolates to nine fungicides of different chemical groups and modes of action, alone and combined with potassium silicate to potentiate their effect with the mycelial growth inhibition method (Table 1), was evaluated in Petri dishes (Fernández-Ortuño, 2014; Fillinger and Walker, 2016).

Preparation of modified culture media and *B. cinerea* sowing

A modified PDA culture medium was used with fungicides alone and combined with potassium silicate; they were emptied into Petri dishes of 100×15 mm. For azoxystrobin, hydroxamic salicylic acid was added at a concentration of 100 ppm to inhibit the alternate oxidative respiration; a disc of 5 mm of PDA was sown with seven-day-old *B. cinerea* growth and incubated in the darkness at 20±1 °C.

Determination of percentage of inhibition

The diameter of the colony was measured every 24 h during seven days in two perpendicular directions using a digital Vernier (Truper[®], Mexico) and it was used to calculate the growth inhibition percentage (GIP) with the Abbot (1925) formula:

$$GIP = \left(\frac{Dc - Dt}{Dc} \right) \times 100$$

where: *GIP*: radial growth inhibition percentage; *Dc*: diameter of the control; *Dt*: diameter of the treatment.

Experimental design

A completely randomized design with six repetitions and two controls was used, where each Petri dish was a repetition. The experiment was repeated twice.

Data analysis

An ANOVA was carried out and a multiple means comparison test with the data obtained, using Tukey's honest significance difference method with a level of significance of 5% in the SAS V.9.1 software for Windows.

RESULTS AND DISCUSSION

Sensitivity of *B. cinerea* to fungicides alone and combined with potassium silicate

According to the statistical analysis, differences were found ($\alpha=0.05$) ($Pr \leq F=0.0001$) between treatments in the mycelial growth diameter (MGD) and the GIP of *B. cinerea* isolated from the four sampling sites, when the fungicides were evaluated alone and when they were combined with potassium silicate.

For the CITAP site, based on the means comparison analysis, it was found that fluazinam was statistically lower in MGD (0 mm) and statistically higher in GIP (100%) than in the rest of the treatments, and azoxystrobin had the highest MGD (100 mm) and showed the lowest GIP (0%) compared to the rest of the treatments (Table 1). When the fungicides were combined with potassium silicate, based on the means comparison analysis, it was identified that fluazinam plus potassium silicate and azoxystrobin plus potassium silicate were statistically lower in MGD (0 mm) and higher in GIP (100%) than the rest of the treatments, while potassium silicate presented the highest MGD (50.16 mm) and the lowest GIP (49.84%) compared to the rest of the treatments (Table 1).

For the Paso del Cristo site, when the fungicides were applied alone, with the means comparison, it was found that fluazinam was statistically lower in MGD (0 mm) and statistically higher in GIP (100%) than the rest of the treatments, and boscalid and azoxystrobin presented the highest MGD (100 mm) and the lowest GIP (0%) compared to the rest of the treatments (Table 1). In the combination of fungicides plus potassium silicate, based on the means comparison analysis, it was seen that the fungicides fluazinam, azoxystrobin and iprodione, all in combination with potassium silicate, were statistically lower than the rest of the treatments in MGD with a value of 0 mm and higher in GIP (100%), while potassium silicate presented the highest MGD (52 mm) and lowest GIP (48%) compared to the rest of the treatments (Table 1).

In the Sierra Negra 1 site, in the treatments with fungicides alone, based on the means comparison analysis, it was found that iprodione was statistically lower in MGD (0 mm) and higher in GIP (100%) than the rest of the treatments and azoxystrobin presented the highest MGD (100 mm) and the lowest GIP (0%) compared to the rest of the treatments (Table 1). In the treatments where the fungicides were combined with potassium silicate, the means comparison analysis found that fluazinam, azoxystrobin and iprodione in combination with potassium silicate were statistically lower in MGD (0 mm) than the rest of the treatments, and higher in GIP (100%) than the rest of the treatments, while fludioxonil showed the highest MGD with a value of 51.5mm and lowest GIP (48.5%) compared to the rest of the treatments (Table 1).

In the Sierra Negra 2 site, based on the means comparison analysis, it was identified that fluazinam was statistically lower in MGD (0 mm) and higher in GIP (100%) than the rest of the treatments, while boscalid and azoxystrobin presented the highest MGD (100 mm) and lowest GIP (0%) compared to the rest of the treatments (Table 1). When combining with potassium silicate, the means comparison analysis showed that fluazinam and azoxystrobin plus potassium silicate were statistically lower in MGD (0 mm) and higher in GIP (100%) than the rest of the treatments and potassium silicate alone, and presented the highest MGD (55.5 mm) and lowest GIP (44.5%) compared to the rest of the treatments (Table 1).

The sensitivity of nine fungicides of different chemical group and mode of action (Fillinger and Walker, 2016) alone and in combination with potassium silicate was evaluated, with the principle of mycelium inhibition (Fernández-Ortuño, 2014); in this regard, Shao *et al.* (2015) found five isolates of *B. cinerea* resistant to fluazinam associated to mutations, the values of CE₅₀ varied from 0.23 to 0.44 $\mu\text{g mL}^{-1}$. Based on this study, the four isolates used

Table 1. Mycelial growth diameter (MGD) and growth inhibition percentage (GIP) of *B. cinerea* isolated from four sampling sites controlled with nine fungicides alone and in combination with potassium silicate.

Fungicides alone									
Fungicide	Concentration $\mu\text{g mL}^{-1}$	CITAP		Paso del Cristo		Sierra Negra 1		Sierra Negra 2	
		MGD (mm)	GII (%)	MGD (mm)	GII (%)	MGD (mm)	GII (%)	MGD (mm)	GII (%)
Fluazinam	0.08	0i*	100a	0h	100a	15.33g	84.67b	0h	100a
Fenhexamid	0.01	71.17c	28.83g	69.83b	30.17g	65c	35f	74.5b	25.5g
Tiofanato metil	0.15	38.18f	61.83d	36.33e	63.67d	42.17e	57.83d	37.67e	62.33d
Captan	3.5	47.83e	52.16e	43.5d	56.5e	51.67d	48.33e	44.83d	55.17e
Pirimethanil	0.08	29.33g	70.67c	25.33f	74.67c	31.5f	68.5c	26.5f	73.5c
fludioxonil	0.004	60d	40f	56.33c	43.67f	64c	36f	59.83c	40.17f
Boscalid	0.4	80.17b	19.83h	100a	0h	91.33b	8.67g	100a	0h
Azoxystrobin	0.2	100a	0i	100a	0h	100a	0h	100a	0h
Iprodiona	0.15	15.5h	84.5b	14g	86b	0h	100a	15.67g	84.33b
LSD		2.78	2.78	3.25	3.25	1.99	1.99	1.8	1.8
Fungicides plus potassium silicate (500 $\mu\text{g mL}^{-1}$)									
Fluazinam	0.08	0g	100a	0f	100a	0g	100a	0h	100a
Fenhexamid	0.01	28.67d	71.33d	51.83a	48.17f	50.33a	49.67g	32d	68e
Tiofanato metil	0.15	27.5d	72.5d	24.67d	75.33c	33.67d	66.33d	25.67e	74.33d
Captan	3.5	35.17c	64.83e	35.17c	64.83d	40.83c	59.17e	34.67c	65.33f
Pirimethanil	0.08	22.83e	77.17c	17.33e	82.66b	21.5f	78.5b	17.67f	82.33c
fludioxonil	0.004	44.17b	55.83f	43.67b	56.33e	51.5a	48.5g	49.33b	50.67g
Boscalid	0.4	20.67e	79.33c	36.67c	63.33d	27.17e	72.83c	35.17c	64.83f
Azoxystrobin	0.2	0g	100a	0f	100a	0g	100a	0h	100a
Iprodiona	0.15	10.5f	89.5b	0f	100a	0g	100a	8.5g	91.5b
Silicato de Potasio	500	50.17a	49.83g	52a	48f	47.5b	52.5f	55.5a	44.5h
LSD		3.28	3.28	2.43	2.43	2.49	2.49	1.36	1.36

*The mean values followed by the same letters in the same column are statistically equal (Tukey $\alpha=0.05$) according to Tukey's honest significant difference.

in this study could be considered sensitive to fluazinam; the isolates from the CITAP, Paso del Cristo and Sierra Negra 2 sites presented a GIP of 100%, being the best treatments; the isolate from Sierra Negra 1 presented a GIP of 84.66% at a concentration of 0.08 $\mu\text{g mL}^{-1}$. Few cases of resistance to fluzinam have been reported for *B. cinerea* (Fillinger and Walker, 2006). Regarding the fungicide Fenhexamid, Fernandez-Ortuño *et al.* (2014), in strains of *B. cinerea* isolated from strawberries, obtained a CE_{50} between the discriminatory doses of 25 and 100 $\mu\text{g mL}^{-1}$ and the isolates with a $\text{CE}_{50} \geq 50 \mu\text{g mL}^{-1}$ were considered resistant. On the other hand, Esteriol *et al.* (2017) reported different degrees of resistance associated to mutations in the isolates of *B. cinerea* from grapevine, with a $\text{CE}_{50} \geq 1.25$ to 299 $\mu\text{g mL}^{-1}$. Based on these studies, the isolates used in this study could be considered sensitive to fenhexamid since they presented GIP of 25.5% (Sierra Negra 2) to 35% (Sierra Negra 1) at a concentration of 0.01 $\mu\text{g mL}^{-1}$. Studies that define the base line of sensitivity

of *B. cinerea* to methyl thiophanate showed that isolates with values of CE_{50} higher than $50 \mu\text{g mL}^{-1}$ are resistant. Based on a discriminatory dose of $50 \mu\text{g mL}^{-1}$, Mercier *et al.* (2010) found that 92% of the isolates were resistant to methyl thiophanate in strawberry fields of California, USA, and based on these criterion the isolates of this study could be considered sensitive, because their growth was inhibited with $0.15 \mu\text{g mL}^{-1}$ presenting a GIP of 57.83% (Sierra Negra 1) to 63.66% (Paso del Cristo). Resistance to multi-site fungicides has been observed only in some cases in *Botrytis* spp., and it seems to imply the detoxification (Fillinger and Walker, 2016). In this study it was found that the four isolates evaluated can be considered sensitive to captan since they presented GIP that varied from 48.33 % (Sierra Negra 1) to 56.5 % (Paso del Cristo), with a concentration of $3.5 \mu\text{g mL}^{-1}$. Esteriol *et al.* (2017) detected that five out of 10 isolates of *B. cinerea* obtained from grapevine showed resistance to pyrimethanil with values of CE_{50} from 10.2 to $62.1 \mu\text{g mL}^{-1}$, associated with mutations, and based on these studies 100% of the isolates from this study could be considered sensitive because they presented a GIP that varied from 68.5% for the Sierra Negra 1 site to 74.66% for Paso de Cristo with a concentration of $0.08 \mu\text{g mL}^{-1}$. Fernandez-Ortuño *et al.* (2014) obtained a CE_{50} in isolates of *B. cinerea* obtained from strawberry between the discriminatory doses of 0.5, 1 and $2 \mu\text{g mL}^{-1}$ and isolates with $CE_{50} \geq 0.5 \mu\text{g mL}^{-1}$ were considered resistant to fludioxonil; based on this criterion, all the isolates of this study could be considered sensitive since they obtained GIP that ranged from 36% (Sierra Negra 1) to 43.66% (Paso del Cristo) using a concentration of $0.004 \mu\text{g mL}^{-1}$. Esteriol *et al.* (2017) detected that the strains of *B. cinerea* isolated from grapevine that grew between CE_{50} 15.04 to $>1000 \mu\text{g mL}^{-1}$ were resistant to boscalid in different degrees of resistance associated to mutations. Fernandez-Ortuño *et al.* (2014) identified isolates of *B. cinerea* resistant to boscalid at $CE_{50} \geq 75 \mu\text{g mL}^{-1}$, and based on this the isolates from the CITAP and Sierra Negra 1 sites could be considered sensitive since they presented GIP of 19.83% and 8.66%, respectively; however, the isolates of the Paso del Cristo and Sierra Negra 2 sites could be considered resistant because they presented GIP equal to zero, that is, there was no *in vitro* control using a concentration of $0.4 \mu\text{g mL}^{-1}$. Regarding the fungicide iprodione, Myresiotis (2007) in strains of *B. cinerea* isolated from different vegetables, obtained a CE_{50} in the range of 0.1 to $1.42 \mu\text{g mL}^{-1}$ and the isolates with a $CE_{50} \geq 1 \mu\text{g mL}^{-1}$ were considered resistant. Grabke *et al.* (2014) consider that the isolates of *B. cinerea* obtained from strawberry and blackberry were moderately resistant to resistant if they grew at concentrations higher than $5 \mu\text{g mL}^{-1}$ of iprodione; based on the studies mentioned, all the isolates evaluated in this study could be considered sensitive since they presented GIP that fluctuated from 84.33% (Sierra Negra 2) to 100% (Sierra Negra 1) at a concentration of $0.15 \mu\text{g mL}^{-1}$. Esteriol *et al.* (2017) and Yin *et al.* (2012) detected that the isolates of *B. cinerea* from grapevine and apple, respectively, which grew in CE_{50} between 102 to $>1000 \mu\text{g mL}^{-1}$ were highly resistant to azoxystrobin associated to mutations, and based on these data, 100% of the isolates from this study could be considered resistant to azoxystrobin since they presented a GIP of 0% using a concentration of $0.2 \mu\text{g mL}^{-1}$; this strobilurina acts in the complex III of respiration (QoIs), and this category of fungicides presents a high risk of resistance development, since the target which is the cytochrome b is codified by a mitochondrial gene (Villani and Cox, 2014); these results can

be partially explained by the management and the applications that producers make in the areas of origin of the isolates, which can be related not only to the number of applications of fungicides by cycle (15 to 20) but also to the intrinsic biological aspects of the populations of pathogens in this area.

The combination of the nine fungicides with potassium silicate decreased the *in vitro* growth in the four isolates since they presented lower MGD and therefore higher GIP than when the fungicide was used alone, and in fact, possibly resistant isolates were not identified, so it can be said that potassium silicate potentiated the effect of the fungicides; it is thought that Si is toxic and that in presence of Si the fungus cannot adequately absorb the sources of carbon from the culture medium, reducing its mycelial growth rate (Velázquez *et al.*, 2017). This could be the explanation for the low growth values of isolates in presence of potassium silicate and it is possible to hypothesize that it is an effect from toxicity, which increases the effect of fungicides.

CONCLUSIONS

All of the isolates (100%) were sensitive to the fungicides fluazinam, fenhexamid, methyl thiophanato, captan, pyrimethanil, fludioxonil and iprodione, showing different GIP. The isolates obtained from Sierra Negra 1 and CITAP were sensitive to the fungicide boscalid, while Sierra Negra 2 and Paso del Cristo were insensitive with GIP values equal to 0; 100% of the isolates were insensitive to the fungicide azoxystrobin with 0% of GIP; the isolates that were insensitive to boscalid and azoxystrobin could be considered resistant because they grew in the culture medium modified with these fungicides and presented 0% of mycelial growth inhibition; however, when these fungicides were combined with potassium silicate, all the isolates were sensitive, and this suggests that the potassium silicate potentiates the effect of fungicides.

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