UNIVERSIDADE FEDERAL DO PARANÁ

JULIANA NICOLAU MAIA

SENSITIVITY OF *Botrytis cinerea* ISOLATES TO FUNGICIDES, DETECTION OF THE PATHOGEN IN TRANSPLANTS AND, EFFICACY OF FUNGICIDES IN THE CONTROL OF STRAWBERRY GRAY MOLD

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Tese apresentada ao Programa de Pós-Graduação em Agronomia, Área de Concentração em Produção Vegetal, Departamento de Fitossanidade e Fitotecnia, Setor de Ciências Agrárias, Universidade Federal do Paraná, como parte das exigências para obtenção do título de Doutora em Ciências.

Orientador: Prof. Dr. Henrique da Silva Silveira Duarte

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação AGRONOMIA (PRODUÇÃO VEGETAL) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **JULIANA NICOLAU MAIA** intitulada: **SENSITIVITY OF *Botrytis cinerea* ISOLATES TO FUNGICIDES, DETECTION OF THE PATHOGEN IN TRANSPLANTS AND, EFFICACY OF FUNGICIDES IN THE CONTROL OF STRAWBERRY GRAY MOLD** , sob orientação do Prof. Dr. HENRIQUE DA SILVA SILVEIRA DUARTE, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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‘’A tarefa não é tanto ver
aquilo que
ninguém viu, mas pensar o
que ninguém ainda pensou sobre
aquilo que todo mundo vê.’
Arthur Schopenhauer

RESUMO

O cultivo do morangueiro é de grande importância no estado do Paraná, que é o terceiro maior produtor do Brasil. Porém, aumento da produção fica frequentemente limitado pelos problemas fitossanitários que acometem a cultura, um dos principais é o mofo cinzento, causado por *Botrytis cinerea.* Devido à dificuldade de controle desta doença e a necessidade dos produtores em importar mudas este estudo teve como objetivos avaliar fungicidas que podem contribuir no manejo do mofo cinzento do morangueiro e avaliar os riscos de entrada de isolados de *Botrytis* de diferentes fenótipos/espécies pela importação de mudas no cultivo do morangueiro no Brasil. A tese está dividida em 3 capítulos e os objetivos específicos de cada capítulo desta tese foram: i) Estabelecer a sensibilidade basal de isolados de *Botrytis cinerea* coletados em 2017 no Paraná à ciprodinil e fludioxonil e avaliar eficácia de controle; ii) avaliar a sensibilidade de isolados de *B. cinerea* do estado do Paraná ao pirimetanil, avaliando possível resistência cruzada com ciprodinil e a sua eficácia de controle; iii) avaliar a incidência de *Botrytis spp*. em mudas importadas, identificando as espécies de *Botrytis* vindo das mudas e avaliando a sensibilidade desses isolados aos fungicidas utilizados no manejo do morangueiro no estado do Paraná. A mistura dos fungicidas fludioxonil + ciprodinil foi registrada para o morangueiro no Brasil em 2019 e ainda não foi amplamente utilizada no país. Os valores de CE_{50} para fludioxonil variaram de 0,0028 to 0,0349 µg/ml já para ciprodinil variaram de 0,0035 to 77,17 µg/mL, tanto fludioxonil quanto ciprodinil, obtiveram bons resultados de eficácia de controle nos ensaios ex vivo 98,3% e 77,34%, respectivamente. O fungicida pirimetanil ainda é pouco utilizado no estado, apesar de ter registro desde 2002, os valores de CE_{50} de *B. cinerea* para pirimetanil variaram de 0,0052 a 94,07 µg/ml e sua eficácia foi de 80,86% mostrando que ainda é importante no manejo do mofo cinzento. A correlação de resistência cruzada entre pirimetanil e ciprodinil foi positiva 0.83, que significa que isolados resistentes a pirimetanil também apresentam resistência a ciprodinil. No que se refere as mudas, todos os isolados foram classificados como *Botrytis cinerea*, mostrando que por enquanto novas espécies não estão chegando ao país por meio das mudas. Apesar de não apresentar novas espécies vindas com as mudas, a presença de *B. cinerea* foi frequente, o que mostra que as mudas importadas são uma fonte importante de inóculo primário. A porcentagem de isolados sensíveis a procimidona, fludioxonil iprodiona, ciprodinil, pirimetanil, boscalida e fluazinam foi de 86,1; 89,9; 81; 21,5; 21,5; 38 e 94,9 %, respectivamente. Em conclusão a mistura de fungicida fludioxonil+ciprodinil recentemente registrada para o controle de doenças do morangueiro pode ser incorporada no manejo de doenças da cultura, pois além dos bons resultados in vitro apresentaram alta eficácia de controle da doença. Pirimetanil também pode ser utilizado no controle do mofo cinzento, por ainda apresentar alta eficácia de controle da doença. Embora não esteja entrando novas espécies de *Botrytis* por meio das mudas importadas, elas são uma importante fonte de inóculo primário e a entrada de isolados com baixa sensibilidade aos fungicidas ciprodinil e pirimetanil, representam risco para produção e este fato deve ser considerado no manejo da doença. Portanto é importante seguir acompanhando a entrada desses isolados e suas sensibilidades aos fungicidas.

Palavras-chave: Controle químico; manejo de doenças de plantas; mofo cinzento; mudas.

ABSTRACT

Strawberry cultivation is of great importance in the state of Paraná, the third largest producer in Brazil. However, the increase in production is often limited by phytosanitary problems that affect the crop, one of the main ones being gray mold, caused by *Botrytis cinerea*. Due to the difficulty of controlling this disease and the need for growers to import transplants, this study aims to evaluate fungicides that can contribute to the management of strawberry gray mold and to evaluate the risks of entry of *Botrytis* isolates of different phenotypes/species in imported strawberry transplants in Brazil. The thesis is divided into 3 chapters and the specific objectives of each chapter of this dissertation were: i) To establish the baseline sensitivity of *Botrytis cinerea* isolates collected in 2017 in Paraná to cyprodinil and fludioxonil and to evaluate the control efficacy; ii) evaluate the sensitivity of *B. cinerea* isolates from the state of Paraná to pyrimethanil, evaluating possible cross-resistance with cyprodinil and its control efficacy; iii) to evaluate the incidence of *Botrytis sp*p. in imported transplants, identifying the *Botrytis* species coming from the transplants and evaluating the sensitivity of these isolates to the fungicides used in strawberry management in the state of Paraná. The mixture of fludioxonil + cyprodinil fungicides was registered for strawberry in Brazil in 2019 and has not yet been widely used in the country. EC_{50} values for fludioxonil ranged from 0.0028 to 0.0349 µg/ml, while for cyprodinil they ranged from 0.0035 to 77.17 µg/ml. Both fludioxonil and cyprodinil obtained good control efficacy results in ex vivo assays 98.3% and 77.34%, respectively. The fungicide pyrimethanil is still not often used in the state, despite having been registered since 2002. The EC_{50} values of *B. cinerea* for pyrimethanil ranged from 0.0052 to 94.07 µg/ml and its efficacy was 80.86%, showing that is still important in the management of gray mold. The cross-resistance correlation between pyrimethanil and cyprodinil was positive (0.83), which means that pyrimethanil-resistant isolates also show resistance to cyprodinil. Regarding transplants, all isolates were classified as *Botrytis cinerea*, showing that new species are not entering the country through transplants. Despite not presenting new species, *B. cinerea* was frequently found in imported transplants, which shows that they are an important source of primary inoculum. The percentage of isolates sensitive to procymidone, fludioxonil, iprodione, cyprodinil, pyrimethanil, boscalid, and fluazinam was 86.1, 89.9, 81, 21.5, 21.5, 38 and 94.9%, respectively. Based in our findings, the fludioxonil + cyprodinil fungicide mixture recently registered for the control of strawberry diseases can be incorporated in the management of diseases in the crop, since, in addition to the good in vitro results, they presented high efficacy in controlling the disease. Pyrimethanil can also be used to control gray mold, as it still has high disease control efficacy. Although new species of *Botrytis* are not entering through imported transplants, they are an important source of primary inoculum and the entry of isolates with low sensitivity to cyprodinil and pyrimethanil fungicides, represent a risk for production and it-should be considered in the management of the disease. Therefore, it is important to continue monitoring the entry of these isolates and their sensitivity to fungicides.

Keywords: Chemical control; gray mold; plant disease management; transplants.

SUMMARY

1 GENERAL INTRODUCTION

The world's largest strawberry producer is China with a production of more than 3,000,000 t/ha, while Brazil ranks 11th and produced 165,440 t/ha of the fruit in 2020 (ANTUNES, 2021). The state of Paraná is the third strawberry producing state in the country, with 21,450 tons of fruit harvested in 2020 and a planted area of 650 hectares, the second largest in the country (ANTUNES, 2021).

Strawberry production is limited by the many phytosanitary problems that affect its cultivation. Among these problems, diseases stand out, which can cause losses throughout fruit production (BAGGIO et al., 2018). Gray mold, caused by *Botrytis cinerea*, is one of the most important diseases causing significant pre- and post-harvest damage due to flower and fruit infections (DEAN et al., 2012; BAGGIO et al., 2018).

The use of fungicides has been the main strategy used to control strawberry diseases, however, *B. cinerea* has a high risk of resistance, due to its great genetic variability, adaptability, and abundant production of spores (BARDAS et al., 2010). In addition, inadequate management, including successive applications of fungicides with the same active ingredient, leads to increased selection pressure for resistant populations of *B. cinerea* (ZUNIGA et al., 2020). In recent years, isolates of *B. cinerea* resistant to several fungicides have been reported worldwide including azoxystrobin (ISHII et al., 2009), boscalid (BARDAS et al., 2010; FERNANDEZ-ORTUÑO et al., 2012; HABIB et al., 2020; CUI et al., 2021), procymidone (SUN et al., 2010; LIU et al., 2016), iprodione (GRABKE et al., 2014; COSSEBOOM and HU, 2021), cyprodinil (FERNANDEZ-ORTUÑO et al., 2013; COSSEBOOM and HU, 2021), and fludioxonil (LI et al., 2014; SANG et al., 2018; ZHOU et al., 2020; DOWLING et al., 2021). In Brazil, studies have already been carried out on the sensitivity of *B. cinerea* isolates to the main fungicides registered for the crop. Isolates from São Paulo, Espírito Santo, Minas Gerais, and Bahia were resistant to iprodione, procymidone, azoxystrobin and thiophanate-methyl (LOPES et al., 2017; BAGGIO et al., 2018 Isolates resistant to iprodione, procymidone, fluazinam, thiophanate-methyl, azoxystrobin, difenoconazole and boscalid were found in the state of Paraná (MAIA et al., 2021). For this reason and because of the limited number of products registered for the culture (AGROFIT, 2022), growers have been reporting difficulty in controlling the disease, with most of these fungicides not showing good efficacy in the field.

With the increasing selection of isolates resistant to traditionally used fungicides, it is necessary to incorporate new fungicides with different modes of action to reduce selection pressure. In 2019, the mixture of fludioxonil + cyprodinil was registered for use in the management of strawberry diseases in Brazil. This product contains two active ingredients from two different classes of fungicides, phenylpyrrole (fludioxonil) and anilinopyrimidine (cyprodinil). Both active ingredients are site-specific inhibitors and therefore prone to resistance (FRAC, 2022). The rapid selection of resistant isolates reinforces the importance of carrying out anti-resistance management, starting with monitoring the pathogen population before initiating the inclusion of a new fungicide in the management of a disease. Therefore, it is extremely important to determine the baseline sensitivity of the isolates to monitor the selection of resistant populations of pathogens in the years following their use (RUSSEL, 2004; GAO et al., 2017). In addition to baseline sensitivity studies, it is important to evaluate the control efficacy of a new fungicide compared to the fungicides used so it can be incorporated into disease management.

Another option to be explored in the management of gray mold is the use of a fungicide with little use in the state, such as pyrimethanil. However, *B. cinerea* has a medium inherent risk of developing resistance to anilinopyrimidines (HILBER & HILBER-BODMER, 1998). There have already been reports of reduced sensitivity and loss of effectiveness in controlling gray mold on grapes in Switzerland and France (HILBER & SCHUEPP, 1996; HILBER & HILBER-BODMER, 1998; LEROUX et al., 1999). In the state of Paraná, sensitivity studies have not yet been carried out for this fungicide, nor its possible cross-resistance with cyprodinil, which is a fungicide of the same chemical group.

The transplants produced in Brazil present plant health problems, and thus growers choose to import transplants from Argentina, Chile, and Spain (ANTUNES & COCCO, 2012; COSTA et al., 2018). The problem is that transplants can be an important source of primary inoculum and transport the pathogen through latent infections to commercial strawberry fields, as has already been observed in the USA (OLIVEIRA et al., 2013; 2017). Different subpopulations of *Botrytis* spp. may be selected in nurseries producing transplants under different fungicide spraying conditions (AMIRI et al., 2018).

Although there are no reports of other species infecting strawberries in countries where Brazil has imported transplants (FARR & ROSSMAN, 2021), *B. pseudocinerea* has already been reported causing gray mold in vineyards in Spain (ACOSTA MOREL et al., 2018). In Chile, the presence of *B. pseudocinerea* in peonies was reported (MUÑOZ et al., 2016). This species has already been reported causing gray mold on strawberries in Germany (LEROCH et al., 2013; PLESKEN et al., 2015).

In addition to the entry of new species when importing transplants, we may also import isolates with resistance to the main fungicides used in the country. In the United States, *B. cinerea* isolates from strawberry transplants were mostly resistant to 4 fungicides used to manage strawberry diseases (OLIVEIRA et al., 2017). Studies with imported strawberry transplants and the incidence of *Botrytis* have not been performed in Brazil.

Based on this information, the objectives of this study were: i) to establish the baseline sensitivity of *Botrytis cinerea* isolates collected in 2017 in Paraná to cyprodinil and fludioxonil and to evaluate control efficacy of these fungicides; ii) evaluate the sensitivity of *B. cinerea* isolates from the state of Paraná to pyrimethanil and its possible cross-resistance with cyprodinil, and evaluate its control efficacy; iii) to evaluate the incidence of *Botrytis* spp. in imported transplants, identify the *Botrytis* species coming from the transplants and evaluate the sensitivity of these isolates to the fungicides iprodione, procymidone, boscalid, fluazinam, fludioxonil, cyprodinil.

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3 CHAPTER 1: Baseline sensitivity of *Botrytis cinerea* strawberry isolates from Paraná state, Brazil to fludioxonil and cyprodinil and disease control efficacy[1]

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Abstract

Botrytis cinerea is an important pathogen in strawberry. The disease (gray mold) is difficult to control, causing losses in the field and during post-harvest. Chemical control is the most used method against the disease, but there are several reports of resistance to fungicides worldwide. The mixture of fungicides fludioxonil + cyprodinil was registered for strawberry in Brazil in 2019 but has not yet been widely used in the country. The objectives of this work were to evaluate the baseline sensitivity of *B. cinerea* isolates to fludioxonil and cyprodinil and the efficacy of these fungicides in the control of post-harvest gray mold. In this study, the baseline sensitivity of *B. cinerea* isolates to fludioxonil and cyprodinil was established based on the EC_{50} of 100 isolates collected in the state of Paraná in 2017. The mean EC_{50} value for fludioxonil was 0.0118 ± 0.0069 µg/ml and EC_{50} values for cyprodinil ranged from 0.0035 to 77.17 µg/ml. Three control efficacy trials were performed, with preventive and curative applications of detached

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strawberry fruit. The first experiment compared the control efficacy of fludioxonil to procymidone and fluazinam; the second compared cyprodinil to procymidone and fluazinam; and the third compared fludioxonil and cyprodinil with their mixture. Fludioxonil showed a control efficacy of 100% when applied preventively and 96.5% curatively, superior to procymidone and fluazinam; for cyprodinil, the highest efficacy was 85.7% in the preventive application. Fludioxonil and the mixture fludioxonil + cyprodinil had better efficacy preventively; however, curatively, fludioxonil was the most effective. These results indicate that the mixture of fludioxonil and cyprodinil fungicides is effective in controlling the disease, with fludioxonil being the most effective fungicide, both preventively and curatively. Thus, this fungicide mixture can be useful in the integrated management of gray mold of strawberry in Brazil.

Keywords: Anilinopyrimidine; Chemical control; Plant diseases; Phenylpyrrole.

Introduction

Botrytis cinerea Pers.:Fr. (teleomorph: *Botryotinia fuckeliana* (de Bary) Whetzel) is the causal agent of gray mold, an important pre- and postharvest disease of strawberry (Williamson et al. 2007). The disease is highly destructive and can spread quickly in the field, reducing yield, and rendering the fruit unmarketable (Dean et al. 2012). The use of fungicides has been the main strategy for disease control worldwide (Vitale et al. 2016). However, *B. cinerea* has a high risk of developing resistance, due to its great genetic variability, adaptability, and abundant spore production (Bardas et al. 2010). In addition, inadequate management, including successive applications of fungicides with the same active ingredient/mode of action, lead to increased selection pressure for resistant populations of *B. cinerea* (Zuniga et al. 2020). In recent years, isolates of *B. cinerea* resistant to various fungicides have been reported worldwide: azoxystrobin (Ishii et al. 2009), boscalid (Bardas et al. 2010; Fernandez-Ortuño et al. 2012; Habib et al. 2020; Cui et al. 2021), procymidone (Sun et al. 2010; Liu et al. 2016), iprodione (Grabke et al. 2014; Cosseboom e Hu 2021), cyprodinil (Fernandez- Ortuño et al. 2013; Avenot et al. 2018; Cosseboom e Hu 2021) and fludioxonil (Li et al. 2014; Sang et al. 2018; Zhou et al. 2020; Dowling et al. 2021).

In Brazil, the sensitivity of *B. cinerea* isolates to the main fungicides registered for the crop has already been studied. Isolates from São Paulo, Espírito Santo, Minas Gerais, and Bahia were resistant to iprodione, procymidone, azoxystrobin and thiophanate-methyl (Lopes et al. 2017; Baggio et al. 2018). In the state of Paraná, isolates resistant to iprodione, procymidone, fluazinam, thiophanate methyl, azoxystrobin, difenoconazole and boscalid were found (Maia et al. 2021). For this reason, in addition to the lack of efficacious alternative disease management measures, growers have been reporting difficulties with disease control. Most registered fungicides have not been demonstrating good efficacy in the field. Therefore, the registration and use of new products is warranted but should be associated with monitoring the sensitivity of the pathogen population, to prolong their efficacy in the field, and avoid early control failure.

With the increasing selection of isolates resistant to commonly used fungicides, it is necessary to incorporate new fungicides with different modes of action to reduce selection pressure. In 2019, the mixture of fludioxonil + cyprodinil was registered for the management of strawberry diseases in Brazil. This product contains two active ingredients from two different classes of fungicides, phenylpyrrole (FRAC#12) (fludioxonil) and anilinopyrimidine (FRAC#9) (cyprodinil). Both active ingredients are site-specific inhibitors and therefore prone to resistance (FRAC 2022). Fludioxonil inhibits phosphorylation of transport-associated proteins and glycerol synthesis by blocking the transmission of osmotic signals (Lew 2010). This fungicide is highly efficacious in inhibiting the mycelial growth of phytopathogenic fungi (Errampalli 2004;

Zhao et al. 2010; Gao et al. 2017). Cyprodinil inhibits the biosynthesis of methionine and other amino acids (Masner et al. 1994). The anilinopyrimidines cyprodinil, pyrimethanil and mepanipyrim act mainly by inhibiting germ tube elongation and mycelial growth of *B. cinerea* (Hilber and Schuepp 1996; Leroux 1996).

Fludioxonil is classified as a fungicide with low to medium risk of resistance (FRAC 2022). Despite this, resistant isolates have already been reported in field populations of *Alternaria* spp., *Penicillium digitatum*, and *Botrytis cinerea* (Iacomi-Vasilescu et al. 2004; Kanetis et al. 2006; Li et l. 2014). Cyprodinil is classified as a medium-risk fungicide for resistance (FRAC 2022), with resistant *B. cinerea* isolates found two years after the fungicide was introduced in Chilean vineyards (Latorre et al. 2002). The rapid selection of resistant isolates reinforces the importance of carrying out anti-resistance management strategies, starting with monitoring the pathogen population before initiating the inclusion of a new fungicide in the management of a crop. Therefore, it is important to determine the baseline sensitivity of the isolates to monitor the development of resistance to fungicides in populations of pathogens in the years following their use (Russel 2004; Gao et al. 2017). Thus, when an increase in fungicide-resistant isolates is noted compared to the baseline sensitivity, it is still possible to use anti-resistance strategies, such as limiting the number of fungicide applications per season and alternating fungicides with different modes of action (Latorre and Torres 2012), avoiding fungicide inefficaciousness in the field.

In addition to baseline sensitivity studies, it is important to evaluate the control efficacy of a new fungicide compared to the fungicides used so that it can be incorporated into disease management recommendations.

As no studies have been carried out in the country with the active ingredients fludioxonil and cyprodinil in relation to *B. cinerea* isolates from strawberry, the objectives of this study were to establish the baseline sensitivity of *B. cinerea* isolates collected in 2017 in the state of Paraná for the fungicides fludioxonil and cyprodinil; and to evaluate the efficacy of these fungicides in the control of strawberry gray mold on detached fruit tests.

Material and Methods

Isolate collection

A total of 100 *B. cinerea* isolates were obtained from 89 commercial strawberry fields in the main producing regions of the state of Paraná in 2017 (Table 1). All isolates came from symptomatic fruit. The crops had not yet received any spraying with fludioxonil or cyprodinil, which were only registered for use in the crop in 2019 as a pre-mixture (AGROFIT 2022). The isolates were obtained through indirect isolation. Part of the infected tissue inside the fruit was transferred to potato-dextrose-agar (PDA) medium at 22°C with a 12-hour photoperiod for seven days. The fungus was subcultured to obtain pure colonies and monosporic isolations were performed. In order not to lose their characteristics after successive transfers, all isolates were preserved with two different methods: in silica-gel using filter paper disks and in glycerol, both stored at -20ºC. The collection of isolates was deposited at the Laboratory of Epidemiology for Integrated Management of Diseases (LEMID) at the Federal University of Paraná. All 100 isolates obtained from the different producing regions of Paraná were previously identified molecularly as *B. cinerea* (Maia et al. 2021).

Baseline sensitivity of *B. cinerea* isolates to fludioxonil and cyprodinil

The 100 isolates were analyzed for sensitivity to fludioxonil (Maxim®, Syngenta Crop Protection, Monthey, Switzerland) by mycelial growth inhibition assays. Aliquots of stock solution were added to autoclaved Potato Dextrose Agar (PDA) medium cooled to 60º C to obtain fludioxonil-amended media at final concentrations of 0 (control), 0.001, 0.0025, 0.005, 0.01, 0.05 and 0.1 µg/mL of fludioxonil. For cyprodinil (Unix® 750 WG, Syngenta Crop Protection) the sensitivity of the isolates was also evaluated by mycelial growth inhibition assay but in L-asparagine agar medium (Hilber and Schuepp 1996) amended with 0 (control), 0.001, 0, 01, 0.03, 0.1, 1, 10 µg/ml cyprodinil. Mycelium plugs 5-mm in diameter were removed from the margins of the *B. cinerea* colonies and placed in 90 mm petri dishes with the respective media and concentrations. For each isolate, two replicates per concentration were used. The experiment was performed twice. The plates were incubated at 22°C in the dark for 2 days for fludioxonil and 5 days for cyprodinil. For each plate, the mean colony diameter was measured in two perpendicular directions.

Efficacy of preventive and curative application of fludioxonil in the control of gray mold

To evaluate the efficacy of fludioxonil, an experiment was conducted in a double factorial scheme (4 x 2), in a completely randomized design, with eight replications. The factors analyzed were fungicides and timing of application (preventive or curative). The evaluated fungicides were fludioxonil at a concentration of 250 mg/L (µg/ml) a.i.; procymidone (Sumilex 500 WP, Sumitomo) at a concentration of 500 mg/L (µg/ml) a.i., as it is the most widely used fungicide in the state of Paraná and has the same mode of action as fludioxonil; fluazinam (Frowncide® 500 SC, ISK) at a concentration of 500 mg/L a.i. for having shown the best performance in previous trials (Maia et al. 2021) and a control treatment (water). The fungicides were diluted in distilled water to obtain spray suspensions at the concentrations recommended by the manufacturers. In the case of the fungicide fludioxonil, which is not registered alone in the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) for strawberries, 250 mg/L a.i. was used following the same concentration of a.i. in the mixture fludioxonil and cyprodinil, which is registered in MAPA for the culture (AGROFIT 2022). The isolate used was the one with the lowest EC_{50} (concentration to inhibit 50% of the mycelial growth) found in the baseline sensitivity assay (CM011), which was previously characterized as resistant to procymidone and sensitive to fluazinam (Maia et al. 2021). The preventive application of fungicides occurred 24 h before inoculation and the curative application of fungicides occurred 24 h after inoculation. Each replication consisted of five fruit inside a plastic pot 15 cm in diameter and 4.5 cm in height with the presence of sterilized cotton and filter paper moistened with distilled water. Strawberry fruit of the cultivar Albion at the green to red maturation stage, without any fungicide application, were used. These were disinfected with 0.5% sodium hypochlorite for 2 min, washed three times in distilled water and dried at room temperature. Soon after, the fruit used for the preventive treatment were sprayed with the fungicides. After 24 hours, all fruit, for the preventive and curative treatments, were inoculated without wounding with 30 µl of suspension containing 10^5 spores/mL and kept in an incubator at 22°C with a 12-hour photoperiod. The fruit used for curative evaluation were sprayed with fungicides 24 h after inoculation. The incidence (% percentage of fruit with signs of the pathogen) was evaluated on the fifth day after inoculation. The experiment was performed twice.

Efficacy of preventive and curative applications of cyprodinil in the control of gray mold

To evaluate the efficacy of cyprodinil against gray mold on strawberries, an experiment was conducted in a double factorial scheme (4 x 2), in a completely randomized design, with eight replications. The factors analyzed were fungicides and preventive and curative application. The evaluated fungicides were cyprodinil at a concentration of 375 mg/L a.i., procymidone at a concentration of 500 mg/L a.i., fluazinam at a concentration of 500 mg/L a.i. and a control treatment (water). The fungicides were diluted in distilled water to obtain spray mixtures and the concentrations used were those recommended for field application by the manufacturers. In the case of the fungicide cyprodinil, which is not registered for strawberry, the concentration of 375 mg/L of a.i. was used following the concentration of a.i. in the mixture fludioxonil and cyprodinil, which is registered in MAPA for the culture (AGROFIT 2022). The isolate used was the one with the lowest EC_{50} found in the baseline sensitivity assay (SJ07 7), which was previously characterized as procymidone resistant and fluazinam sensitive (Maia et al. 2021). The preventive and curative applications, as well as the repetitions and evaluations were carried out as described in the previous experiment. The experiment was performed twice.

Efficacy of preventive and curative applications of fludioxonil and cyprodinil when compared to their mixture

As only the mixture of fludioxonil and cyprodinil is registered in the MAPA for strawberry (AGROFIT 2022), this experiment was conducted to find out the effect of the fungicides separately compared to the commercially available pre-mixture to obtain information on which fungicide is more efficacious. For this, an experiment was conducted in a double factorial scheme (4 x 2), in a completely randomized design, with eight replications, using an isolate with low EC_{50} for both fungicides. The factors analyzed were fungicides and timing of application (preventive or curative). The evaluated fungicides were fludioxonil at a concentration of 250 mg/L a.i., cyprodinil at a concentration of 375 mg/L a.i.; mixture of fludioxonil at a concentration of 250 mg/L a.i. + cyprodinil at a concentration of 375 mg/L a.i., which is the recommended by the manufacturer, and a control treatment (water). The fungicides were diluted in distilled water to obtain spray mixtures. The isolate used (CM011) showed low EC_{50} for both fungicides in the baseline sensitivity test. The preventive and curative application, as well as the number of replicates and the evaluations, were carried out as described in the previous experiments. The experiment was performed twice.

Data analysis

The EC_{50} values for each isolate and for each fungicide were estimated using the DRC package (version 3.0-1), through non-linear models chosen based on the lower AIC values (better fit). The best fit model was the 4-parameter log logistic model, represented by the equation: $f(x)=c+(d-c)/(1+\exp(b(\log(x)-\log(e))))$. Baseline sensitivity to fludioxonil and cyprodinil was constructed based on the frequency distribution of EC_{50} values for the 100 isolates.

The control efficacy of fungicides was calculated using the formula $\%C = [(D-T)/D]x100$, where D is the incidence of diseased fruit in the control and T is the incidence of diseased fruit in the fungicide treatments. For the disease incidence data, analysis of variance (ANOVA) was performed for each repetition of the experiments in items 2.3, 2.4 and 2.5. Since the mean square of the residue showed a ratio of less than 7:1 (Banzatto and Kronka 2013) between the repetitions of the experiments, a joint analysis was carried out with the repetitions of the experiments. As the analysis did not meet the assumptions, and there were many zeros, all responses were added the constant 1, and the box-cox transformation parameters were checked. In these cases, the transformations obtained were 1/3, 3/4 and 1/4 for the experiments of items 2.3, 2.4 and 2.5, respectively. Subsequently, ANOVA was performed, and the means were compared using the Tukey test with a 5% probability of error. Statistical analyzes were performed using the R environment (R Core Team 2022).

Results

Baseline sensitivity of *B. cinerea* isolates to fludioxonil and cyprodinil

Based on mycelial growth, the EC_{50} values of the 100 *B. cinerea* isolates for fludioxonil ranged from 0.0028 to 0.0349 µg/ml with a mean EC_{50} value of 0.0118 ± 0.0069 µg/ml. The ratio between the highest and lowest EC_{50} value was 12.46. The frequency distribution of EC_{50} values was skewed with a tail to the right (Fig. 1). EC_{50} values for the isolates from the organic fields were 0.0059 and 0.0117 µg/ml.

For cyprodinil, the EC_{50} values of the isolates ranged from 0.0035 to 0.7 µg/ml which corresponds to 71% of the isolates with an average EC_{50} value of 0.036 ± 0.100 µg/ml. The ratio between the highest and lowest EC_{50} value was 200. Frequency distribution of EC_{50} values for cyprodinil is shown in Fig. 2. EC_{50} values for the isolates from the organic fields were 0.0050 and 0.0078 µg/ml. A total of 29% of the isolates showed EC_{50} greater than 0.7 µg/ml, with the highest value being 77.17 µg/ml, and the average EC_{50} value was 3.41 ± 11.3.

Efficacy of preventive and curative application of fludioxonil in the control of gray mold

There was an interaction between fungicide and timing of application (preventive and curative) ($P = 0.00$). Fludioxonil showed the lowest disease incidence both when applied preventively or curatively among all treatments. Disease control efficacy was 100% when applied preventively, and 96.49% for the curative application. For the preventive treatments, procymidone and fluazinam differed from the control, but fluazinam had better efficacy (lower incidence). However, for the curative treatments, procymidone and fluazinam were not different from the control. The only fungicide that had a difference between preventive and curative applications was fluazinam, which was better when applied preventively (16.25% incidence) than curatively (55%) (Table 2).

Efficacy of preventive and curative application of fungicides compared to cyprodinil in the control of gray mold

There was no interaction between the factors fungicide and timing of application in this trial ($P > 0.1$). The fungicides cyprodinil and fluazinam showed better efficacy in controlling gray mold, followed by the fungicide procymidone. All fungicides differed from the non-treated control. Cyprodinil showed 85.71 and 68.96% control efficacy against gray mold in preventive and curative applications, respectively. All fungicides showed better efficacy (lower disease incidence) when applied preventively (Table 3).

Efficacy of preventive and curative applications of fludioxonil and cyprodinil compared to their mixture

There was interaction between factors fungicide and type of application (preventive and curative) ($P = 0.01$). When the fungicides were applied preventively, fludioxonil + cyprodinil (mixture) and fludioxonil alone showed the best efficacy, followed by the fungicide cyprodinil. All fungicides differed from the non-treated control. However, when fungicides were applied curatively, fludioxonil showed better efficacy than the mixture (fludioxonil + cyprodinil), and cyprodinil did not differ from the control. Better preventive and curative efficacy were observed when the fruit were treated with fludioxonil compared to cyprodinil (Table 4). Regarding the timing of application, the fungicide fludioxonil was efficacious both preventively and curatively, but the fungicides fludioxonil + cyprodinil (mixture) and cyprodinil were more efficacious when applied preventively (Table 4).

Discussion

To the best of our knowledge, this is the first study in Brazil that evaluated *Botrytis cinerea* baseline sensitivity to fludioxonil and cyprodinil fungicides. This information is important for monitoring the development of resistance to these fungicides in the years following their initial use. In addition, this study showed that the fludioxonil + cyprodinil mixture proved to be efficacious in controlling strawberry gray mold, with the fungicide fludioxonil being the active ingredient with the best preventive and curative efficacy. Therefore, the mixture showed potential to be a useful tool in the integrated management of gray mold in Brazil.

In general, isolates were more sensitive to fludioxonil than to cyprodinil. EC_{50} ranged from 0.0028 to 0.0349 µg/ml for fludioxonil. The baseline sensitivity values for fludioxonil found in the present study corroborates previous studies carried out in other countries with *B. cinerea* isolates in strawberry, apple, and pear where the average EC_{50} was 0.008; 0.005 and 0.005 µg/ml, respectively (Hilber et al. 1995; Zhao et al. 2010).

EC_{50} ranged from 0.0035 to 77.17 µg/ml for cyprodinil. Sensitive isolates have been described in Greece with EC_{50} ranging from 0.005 to 0.01 µg/ml (Petsikos-Panayotarou et al. 2003). Although most of our isolates showed high sensitivity to cyprodinil, it is more common to find studies where *B. cinerea* isolates show resistance or reduced sensitivity to cyprodinil (Fernandez-Ortuño et al. 2013; Avenot et al. 2018; Cosseboom and Hu 2021). Fernandez-Ortuño et al. (2013) found moderately resistant isolates with an average EC_{50} of 12.9 µg/ml and resistant isolates with an average EC_{50} of 23 µg/ml. The frequency of cyprodinil-resistant isolates, using a discriminatory dose of 4 µg/ml, was 42% in strawberry cultivation in China and 86% in the United States, for isolates from strawberry, blackberry, black raspberry, red raspberry, and grapes (Fan et al. 2017; Cosseboom and Hu 2021). In this study, it was observed that the EC_{50} values of 71% of the isolates ranged from 0.0035 to 0.7 µg/ml representing the range with the highest frequency of isolates (Fig. 2), and 29% of the isolates showed EC_{50} greater than 0.7 reaching up to 77.17 µg/ml, indicating that these isolates may have resistance even without previous contact with cyprodinil. This can be explained by the registration and use of pyrimethanil, a fungicide registered in Brazil in 2002 (AGROFIT 2022) for strawberry that also belongs to the chemical group of anilinopyrimidines (FRAC 2022).

Isolates resistant to pyrimethanil may also be resistant to other active ingredients of the same group, showing cross-resistance (Hilber and Schuepp, 1996; Myresiotis et al. 2007). Complementary correlation studies of the EC_{50} values of the isolates for cyprodinil and pyrimethanil still need to be carried out, to verify possible cross-resistance of our isolates. In Chilean vineyards, a frequency of 38.5% of cyprodinil-resistant isolates was observed after four applications in only 2 years (Latorre et al. 2002). After seven years of using cyprodinil to control *B. cinerea* in Greece, 57% of isolates were highly resistant (Myresiotis et al. 2007). Another aggravating factor is that studies show that, unlike fludioxonil, isolates with moderate and high resistance to anilinopyrimidines are as competitive as sensitive isolates, and do not show fitness costs (Bardas et al. 2008; Avenot et al. 2018).

Efficacy of fludioxonil and cyprodinil fungicides were compared to procymidone, fluazinam and the fludioxonil + cyprodinil mixture. Fludioxonil exhibited high preventive and curative efficacy in controlling gray mold in our study. Zhao et al. (2010) also demonstrated 100% efficacy of fludioxonil in the control of gray mold on detached fruit of apple and pear. In previous studies, fluazinam presented the best efficacy among different fungicides in controlling strawberry gray mold (Maia et al. 2021). In this study, we observed that the fungicide cyprodinil showed equal efficacy to fluazinam, however, the fungicide fludioxonil showed superior efficacy to both fungicides in controlling the disease. The cyprodinil + fludioxonil mixture showed preventive control that was equally efficacious as fludioxonil, but when applied curatively, its efficacy was reduced even though the same concentration of product was used. The commercial pre-mixture is formulated as water dispersible granules (WGs) whereas fludioxonil is a suspended concentrate (SC), and also the products have different inert ingredients in their formulations, which can influence the coverage of the fruit.

The fungicide fludioxonil has shown excellent performance in controlling the gray mold in strawberries in our study and several field and *in vitro* studies, demonstrating that fludioxonil is one of the best fungicides against the disease (Kim et al. 2007; Petit et al. 2011). However, care must be taken when incorporating it into disease management, as *B. cinerea* isolates resistant to the fungicide have already been found after only five years of registering fludioxonil for the control of gray mold on tomatoes and cucumbers in China (Sang et al. 2018). Therefore, growers should limit the use of fludioxonil and/or apply it in mixture with other fungicides with different modes of action (Ren et al. 2016). In the case of management of strawberry diseases in Brazil, the commercial product registered is a pre-mixture with cyprodinil, which can help in anti-resistance management. Despite these reports, fludioxonil-resistant isolates are rarely found in the field in the USA (Li et al. 2014). One explanation for the reports of resistance not resulting in practical resistance is the reduced fitness of resistant isolates associated with increased sensitivity to osmotic stress, which is consistent with the mode of action of fludioxonil and has already been demonstrated in some studies (Lew 2010; Sang et al. 2018).

In conclusion, the fludioxonil + cyprodinil mixture can be recommended to strawberry growers due to its efficacy in controlling gray mold both preventively and curatively, but the potential risk of resistance must be considered. Monitoring and fitness studies are recommended in the years following its incorporation in the management of strawberry diseases. The baseline sensitivity results described in this study can serve as a comparison for future monitoring of *Botrytis cinerea* in strawberries for fludioxonil and cyprodinil in Brazil.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Juliana Nicolau Maia, Giovana Beger, Natalia Aparecida Peres, Cristiano Nunes Nesi, Louise Larissa May De Mio and Henrique da Silva Silveira Duarte. The first draft of the manuscript was written by Juliana Nicolau Maia and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations Conflict of interest

The authors declare no competing interests.

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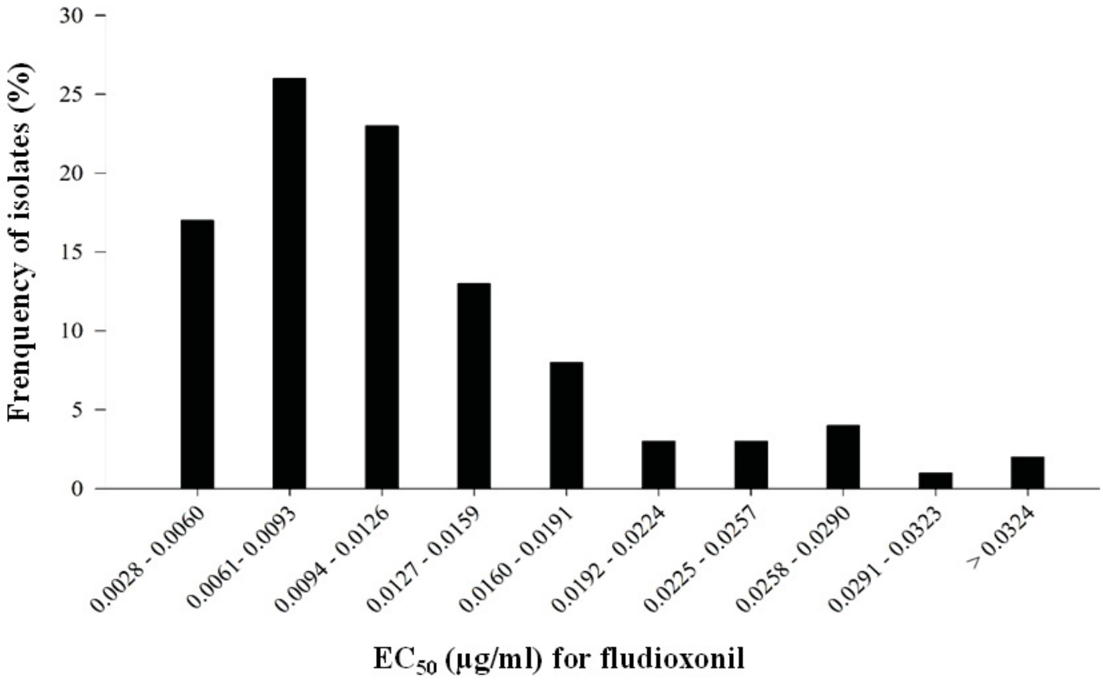

Fig. 1 Frequency distribution of EC_{50} (concentration to inhibit 50% of the mycelial growth) values for fludioxonil from 100 *Botrytis cinerea* isolates collected in different regions of Paraná state, Brazil. Individual EC_{50} values were grouped into class intervals of 0.0032 µg/ml.

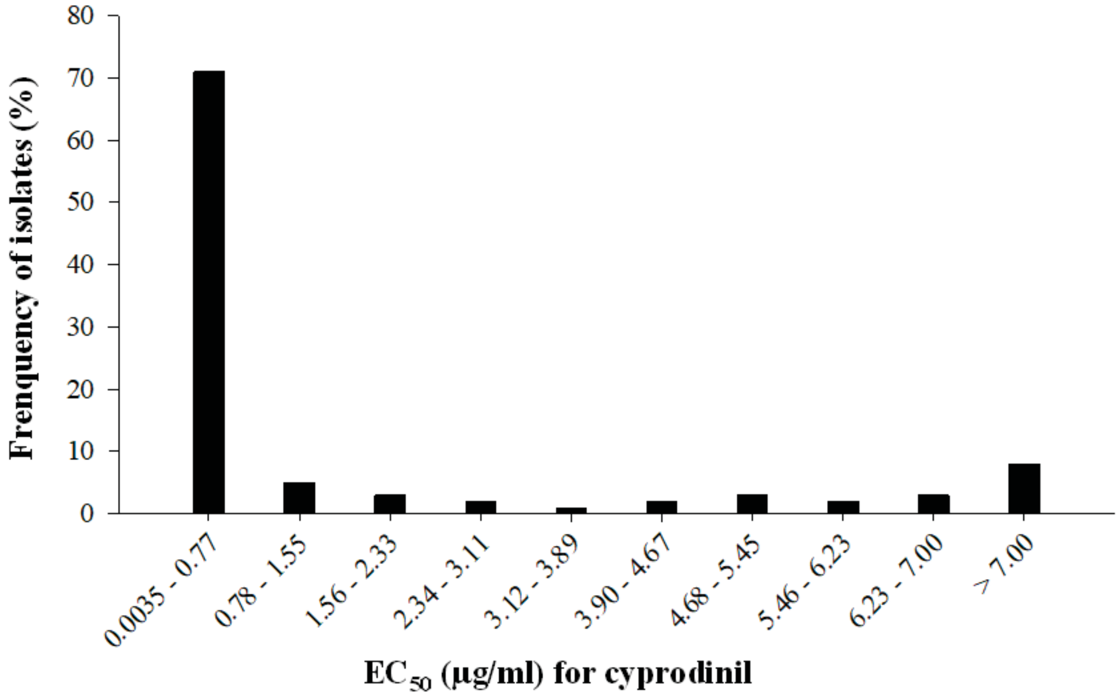

Fig. 2 Frequency distribution of EC_{50} (concentration to inhibit 50% of the mycelial growth) values for cyprodinil from 100 *Botrytis cinerea* isolates collected in different regions of Paraná state, Brazil. Individual EC_{50} values were grouped into class intervals of 0.77 µg/ml.

Table 1 Origin of 100 *B. cinerea* isolates collected from different regions of Paraná state, Brazil

Regions	Municipalities	Number of isolates
Metropolitan region of Curitiba	Araucária	7
	Curitiba	1
	Lapa	1
	*São José dos Pinhais	56
North	Conselheiro Mairinck	4
	Jaboti	9
	Japira	5
	Pinhalão	7
South East	Mallet	2
	Prudentópolis	1
	Rio Azul	5
South West	Francisco Beltrão	1
	**Verê	1

*One isolate from organic production. ** Isolate from organic production

Table 2 Gray mold incidence in strawberry fruits following preventive and curative treatment with the fungicides fludioxonil, fluazinam and procymidone.

Treatment	Disease incidence (%)	
	Preventive	Curative
Control	71.25 Aa*	71.25 Aa
Procymidone	41.25 Ab**	61.25 Aa
Fluazinam	16.25 Bc	55.00 Aa
Fludioxonil	0.00 Ad	2.50 Ab

*Means followed by the same letters, lowercase in the column and uppercase in the row, do not differ, respectively, by the ANOVA and Tukey tests at 5% probability.

**The data were transformed into $(x+1)^{1/3}$ for analysis, however they are presented in the original scale.

Table 3 Gray mold incidence in strawberry fruits following preventive and curative treatment with the fungicides cyprodinil, fluazinam and procymidone.

Treatment	**Disease incidence (%)**		**Average**
	Preventive	**Curative**	
Control	70.00*	72.5	71.25 a
Procymidone	35.00**	47.5	41.25 b
Fluazinam	17.5	33.75	25.62 c
Cyprodinil	10.00	22.50	16.25 c
Average	33.12 B	44.06 A	

*Means followed by the same letters, lowercase in the column and uppercase in the row, do not differ, respectively, by the ANOVA and Tukey tests at 5% probability.

**The data were transformed into $(x+1)^{3/4}$ for analysis, however they are presented in the original scale.

Table 4 Gray mold incidence in strawberry fruits following preventive and curative treatment with the fungicides fludioxonil, cyprodinil and cyprodinil+fludioxonil.

Treatment	**Disease incidence (%)**	
	Preventive	**Curative**
Control	84.17 Aa* **	82.5 Aa
Cyprodinil	37.50 Bb	55.00 Aa
Fludioxonil	0.00 Ac	2.60 Ac
Fludioxonil+Cyprodinil	1.67 Bc	12.50 Ab

* Means followed by the same letters do not differ, respectively, by the ANOVA and Tukey tests at 5% probability

**The data were transformed into $(x+1)^{1/4}$ for analysis, however they are presented in the original scale.

4 CHAPTER 2: Sensitivity of *Botrytis cinerea* isolates from strawberry to the fungicide pyrimethanil in Paraná, Brazil, and its control efficacy[2]

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Abstract

Due to the significant losses caused by the pathogen *Botrytis cinerea* in strawberry, the use of effective management is highly needed but can be difficult to achieve. Chemical control is the main method used against *B. cinerea*, but the pathogen has shown resistance to several fungicides worldwide. The fungicide pyrimethanil is registered for strawberry, but it is still not widely used by growers in Paraná state. The objectives of this study were to evaluate the sensitivity of *B. cinerea* isolates to pyrimethanil, to verify possible cross-resistance between pyrimethanil and cyprodinil, and to evaluate the efficacy of pyrimethanil in controlling gray mold. The sensitivity of *B. cinerea* isolates to pyrimethanil was established based on the EC_{50} of 100 isolates collected in the state of Paraná in 2017. EC_{50} values of *B. cinerea* for pyrimethanil ranged from 0.0052 to 94.07 µg /ml with a mean EC_{50} value of 6.53 ± 15.17 µg/ml. There was a positive correlation

2 This manuscript submitted in Tropical Plant Pathology

between pyrimethanil and cyprodinil EC_{50} values (r = 0.83). The efficacy of preventive and curative applications of pyrimethanil, cyprodinil and fludioxonil was evaluated in detached strawberry fruit assays. Fludioxonil was the most effective in controlling the disease both preventively and curatively. Pyrimethanil and cyprodinil showed greater efficacy when applied preventively, with control efficacy of 84.61 and 87.17%, respectively. Overall, pyrimethanil showed good efficacy in the control of gray mold of strawberry and can be recommended for managing the disease, but its use requires monitoring to avoid the selection of resistant isolates in the short term.

Keywords: Anilinopirimidines; Chemical control; Fragaria x ananassa; Gray mold; Plant disease.

Botrytis cinerea (teleomorph: *Botryotinia fuckeliana*) is a necrotrophic pathogen that infects more than 200 plant species, causing rotting of flowers, leaves and fruit, and resulting in significant yield losses (Williamson et al. 2007). Disease management relies mainly on chemical control. The widespread and repeated use of fungicide groups such as dicarboximides, succinate dehydrogenase inhibitors (SDHIs), anilinopyrimidines, and others has resulted in the selection and predominance of *B. cinerea* isolates resistant to different fungicides in many countries (Fernandez-Ortuño et al. 2012; Habib et al. 2020; Sun et al. 2010; Liu et al. 2016; Fernandez-Ortuño et al. 2013; Avenot et al. 2018; Maia et al. 2021). The widespread distribution of *B. cinerea* isolates with multiple resistance limits the number of fungicides available for efficacious control of the disease (Amiri et al. 2013). Cases of resistance to fungicides in *B. cinerea* are frequently reported due to its high genetic variability, short life cycle, and prolific spore production (Rosslenbroich and Stuebler 2000).

A fungicide registered for gray mold control but not commonly used in the state of Paraná is pyrimethanil, but due to its monogenic resistance, *B. cinerea* has a medium risk of inherent resistance to anilinopyrimidines (FRAC#9) (Hilber and Hilber- Bodmer 1998; FRAC 2022). There have been reports of reduced sensitivity and loss of efficacy in controlling gray mold on grapes in Switzerland and France shortly after its initial release (Hilber and Schuepp 1996; Hilber and Hilber-Bodmer 1998; Leroux et al. 1999). Isolates of B. *cinerea* highly resistant to pyrimethanil have been found in countries such as France, Australia, Spain, Greece, Israel, China, and USA (Leroux et al. 1999; Sergeeva et al. 2002; Petsikos-Panayotarou et al. 2003; Moyano et al. 2004; Sun et al. 2010; Zhao et al. 2010; Korolev et al. 2011). Field isolates of *B. cinerea* resistant to anilinopyrimidine fungicides have also been detected in grapes and vegetables crops (Leroux et al. 1999; Latorre et al. 2002; Baroffio et al. 2003; Moyano et al. 2004). In Brazil, in the states of São Paulo, Minas Gerais, Espírito Santo and Bahia, isolates of *B. cinerea* from strawberry collected from 2013 to 2015 were found resistant to pyrimethanil (Baggio et al. 2018), but no such studies have been performed for Parana state isolates. Due to the similar chemical structures of anilinopyrimidines, cross-resistance between active ingredients may occur (Hilber and Schuepp 1996; Leroux et al. 1999). Therefore, cross-resistance studies and evaluation of control efficacy to generate information for disease management are important.

The objectives of this study were to evaluate the sensitivity of *B. cinerea* isolates from the state of Paraná to pyrimethanil, to correlate its sensitivity with cyprodinil, and to compare the efficacy of preventive and curative applications of pyrimethanil with the fungicides fludioxonil and cyprodinil in the control of gray mold in detached strawberry fruit.

A total of 100 *B. cinerea* isolates were collected from 89 commercial strawberry fields in the main producing regions of the state of Paraná in 2017 (Fig.1), and monosporic isolations were performed. In order not to lose their characteristics after successive transfers, all isolates were stored using two different methods: in silica-gel using filter paper disks, stored at -20ºC, and storage by freezing in glycerol at -20ºC. The collection of isolates is deposited at the Laboratory of Epidemiology for Integrated Management of Diseases (LEMID) at the Universidade Federal do Paraná.

The 100 isolates were evaluated for sensitivity to pyrimethanil (Mythos®, Bayer) by mycelial growth inhibition on L-asparagine agar medium (Hilber and Schuepp 1996) amended with 0 (control); 0.01; 0.05; 0.1; 1; 10; 50 μg/ml pyrimethanil. PDA mycelium plugs 5 mm in diameter were removed from the margins of *B. cinerea* actively growing colonies and placed in 90 mm petri dishes with the respective media and concentrations. For each isolate, two replicates per concentration were used. The experiment was performed twice. The plates were incubated at 22°C in the dark for 5 days. For each plate, the mean colony diameter was measured in two perpendicular directions. The EC_{50} values (effective concentration to inhibit 50% of the mycelial growth) for each isolate were estimated using the DRC package (version 3.0-1), through non-linear models chosen based on the AIC values, in which the lower the AIC, the better the fit. The best suited model was the 4-parameter log logistic model, represented by the equation: $f(x)=c+(d-c)/(1+exp(b(log(x)-log(e))))$. Statistical analyzes were performed using the R software (R Core Team, 2020).

Potential cross-resistance between pyrimethanil and cyprodinil was evaluated using mycelial growth inhibition assays to determine the EC_{50} for cyprodinil, as described above, for the same 100 isolates, but with concentrations of 0; 0.001; 0.01; 0.03; 0.1; 1; 10 μg/ml. EC_{50} values were log-transformed and analyzed to establish correlations. Cross-resistance analysis was performed by calculating Pearson's correlation coefficient (r).

To evaluate the efficacy of pyrimethanil, an experiment was conducted in a double factorial scheme (4 x 2), in a completely randomized design, with eight replications. The analyzed factors were fungicides and timing of application (preventive or curative). The evaluated fungicides were pyrimethanil at a concentration of 600 mg/L a.i.; cyprodinil (Unix 750 WG, Syngenta Crop Protection, Monthey S.A) at a concentration of 375 mg/L a.i., fludioxonil at a concentration of 250 mg/L a.i., and a control treatment (application of water). The fungicides were diluted in distilled water to obtain spray mixtures and the concentrations used were those recommended for field application by the manufacturers. The preventive application of fungicides occurred 24 h before inoculation and the curative application of fungicides occurred 24 h after inoculation. Each replicate consisted of three fruit inside a plastic pot 15 cm in diameter and 4.5 cm in height with cotton and filter paper sterilized and moistened with distilled water to maintain humidity. Strawberry fruit of the cultivar Albion at the green to red maturation stage, without fungicide residues, were used. The fruit were disinfested with 0.5% sodium hypochlorite for 2 min, washed three times in distilled water, and dried at room temperature. Soon after, the fruit used for the preventive treatment were sprayed with fungicides. After 24 hours, all fruit, both for preventive and curative treatments, without wounds, were inoculated with 30 µl of a suspension containing 10^5 spores/mL of the isolate SJ46 4, which had a low EC_{50} value for pyrimethanil. The fruit used for curative treatment were sprayed with fungicides 24 h after inoculation. Fruit were kept at 22°C with a photoperiod of 12 hours. The disease incidence was evaluated on the fifth day after inoculation. The experiment was performed twice.

Control efficacy was calculated using the formula %C = [(D-T) /D]x100, where D is the incidence of diseased fruit in the control and T is the incidence of diseased fruit in the fungicide treatments. Data on fruit disease incidence, and analysis of variance (ANOVA) was obtained for each repetition of the experiment.

Based on mycelial growth inhibition, the EC_{50} values of the 100 *B. cinerea* isolates for pyrimethanil ranged from 0.0052 to 94.07 µg/ml with a mean EC_{50} value of 6.53 ± 15.17 µg/ml. The ratio between the highest and lowest EC_{50} value was 18090.38. Frequency distribution of EC_{50} values is presented in Fig. 2. EC_{50} averages by region of the state of Paraná ranged from 3.12 to 10.95 µg/ml (Table 1). In Greece, all isolates of *B. cinerea* from tomato showed sensitivity to pyrimethanil with EC_{50} ranging from 0.005 to 0.04 µg/ml (Petsikos-Panayotaoru et al. 2003). EC_{50} ranges were used by Amiri et al. (2013) for the classification of isolates, where isolates of *B. cinerea* from strawberry with EC_{50} values lower than 5 µg/ml were considered sensitive to pyrimethanil, whereas isolates with EC_{50} values 5 to 10 and > 10 µg/ml were considered moderately resistant or highly resistant, respectively. According to this classification, 22% of our isolates are highly resistant, 4% moderately resistant, and 74% are sensitive. In the U.S., 24.7% of *B. cinerea* isolates from strawberry were classified as highly resistant to pyrimethanil, with EC_{50} greater than 10 µg/ml and maximum EC_{50} of 125 µg/ml (Amiri et al. 2013). In our study, we found an isolate with an EC_{50} of 94 µg/ml. More recently in China, 72.5% of *B. cinerea* isolates from ginseng were resistant to pyrimethanil, and discontinuing the use of this fungicide to control gray mold was recommended (Wang et al. 2022). In Brazil, in a study using isolates collected from 2013 to 2015 in the states of São Paulo, Minas Gerais, Espírito Santo and Bahia, 12.5% of the isolates collected in conventional strawberry fields had EC_{50} greater than 10 µg/ml (highly resistant), a smaller number than was found in our study (Baggio et al. 2018).

The correlation between the EC_{50} of pyrimethanil and cyprodinil was significantly positive (r = 0.83, *P* = 0.000) (Fig. 3). Our result is like that found in Greece, where the r was 0.71 (Myresiotis et al. 2006) between pyrimethanil and cyprodinil fungicides. However, different from what was found in Amiri et al. (2013), where he observed a moderate positive correlation (r = 0.54), the high correlation found between EC_{50} of cyprodinil and pyrimethanil indicates the occurrence of cross-resistance. *B. cinerea* isolates with low sensitivity to cyprodinil without any previous exposure to the fungicide have been found in the state of Paraná (unpublished data) and this may be explained by the previous use of pyrimethanil in the control of gray mold in the state.

An interaction between fungicide and timing of application (preventive and curative) was observed ($P<0.01$). Fludioxonil showed a lower disease incidence among all treatments when applied both preventively and curatively. When applied preventively, disease control efficacy was 100%, and in the curative application was 97.78% (Table 2). Pyrimethanil and cyprodinil fungicides differed in their efficacy of control for preventive and curative treatments. Both performed better when applied preventively, with an efficacy of 84.61% and 87.17% for pyrimethanil and cyprodinil, respectively. Nevertheless, for preventive and curative treatment, pyrimethanil and cyprodinil fungicides were significantly different than the control. Their curative efficacy was 71.11 and 72.09% for pyrimethanil and cyprodinil, respectively. In another study in Brazil, the efficacy of preventive treatment of pyrimethanil was evaluated using a sensitive isolate, and the incidence of strawberries with gray mold was 14.5% (Baggio et al. 2018), whereas in this study was 12.5%. In Florida, however, there was no incidence of gray mold in fruit treated with pyrimethanil and inoculated with a sensitive isolate (Amiri et al. 2013). Although pyrimethanil showed excellent ex vivo results in our study, the selection of *B. cinerea* isolates resistant to cyprodinil and pyrimethanil is a risk. Four applications per year of cyprodinil for 2 years on a grape orchard in Chile resulted in a high frequency (38.5%) of resistant *B. cinerea* isolates (Latorre et al. 2002). Thus, application of fungicides to control *B. cinerea* should be reduced and anilinopyrimidines should not be used more than three times in a crop cycle. Furthermore, it should always be used in mixture or alternation with other fungicides with different modes of action (Baroffio et al. 2003; Moyano et al. 2004). Therefore, the anti-resistance strategy of using pyrimethanil only once per season remains useful (Sholberg et al. 2005).

In conclusion, the results found in this study are important for monitoring the sensitivity of *B. cinerea* isolates from strawberry in Paraná to the fungicide pyrimethanil. The fungicide was efficacious in controlling gray mold and could be used in the management of the disease. However, potential resistance risks for this group must be considered and applications must be limited and alternated with fungicides from other chemical groups.

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Juliana Nicolau Maia, Giovana Beger, Isadora Brocco Boldrini, Natalia Aparecida Peres, Louise Larissa May De Mio and Henrique da Silva Silveira Duarte. The first draft of the manuscript was written by Juliana Nicolau Maia and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations Conflict of interest

The authors declare no competing interests.

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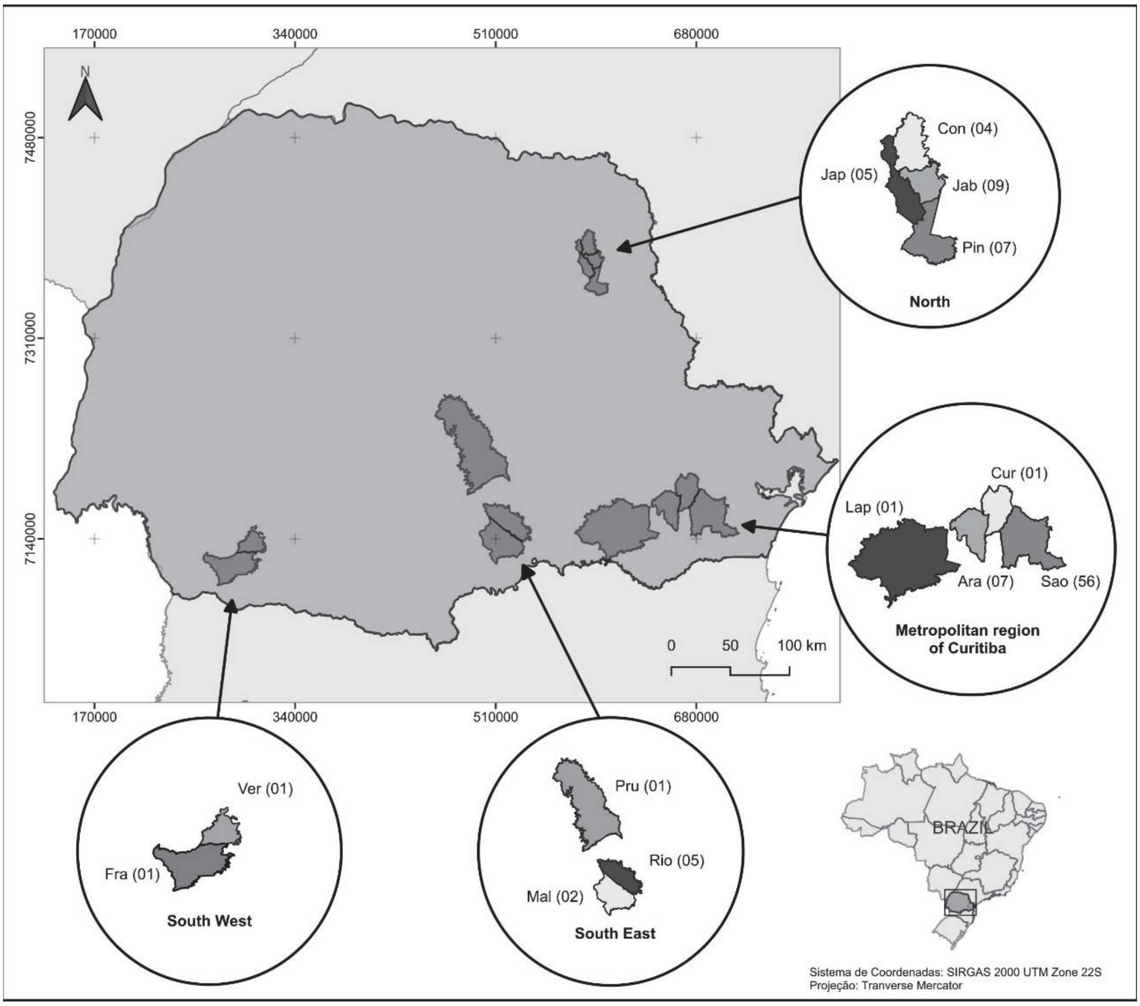

Fig. 1 Regions of collection of strawberry fruit samples in Paraná state, Brazil, in the municipalities of: Araucária (Ara); Conselheiro Mairink (Con); Curitiba (Cur); Francisco Beltrão (Fra); Jaboti (Jab); Japira (Jap); Lapa (Lap); Mallet (Mal); Pinhalão (Pin); Prudentópolis (Pru); Rio Azul (Rio); São José dos Pinhais (Sao) and Verê (Ver). The numbers represent the number of samples collected by municipality.

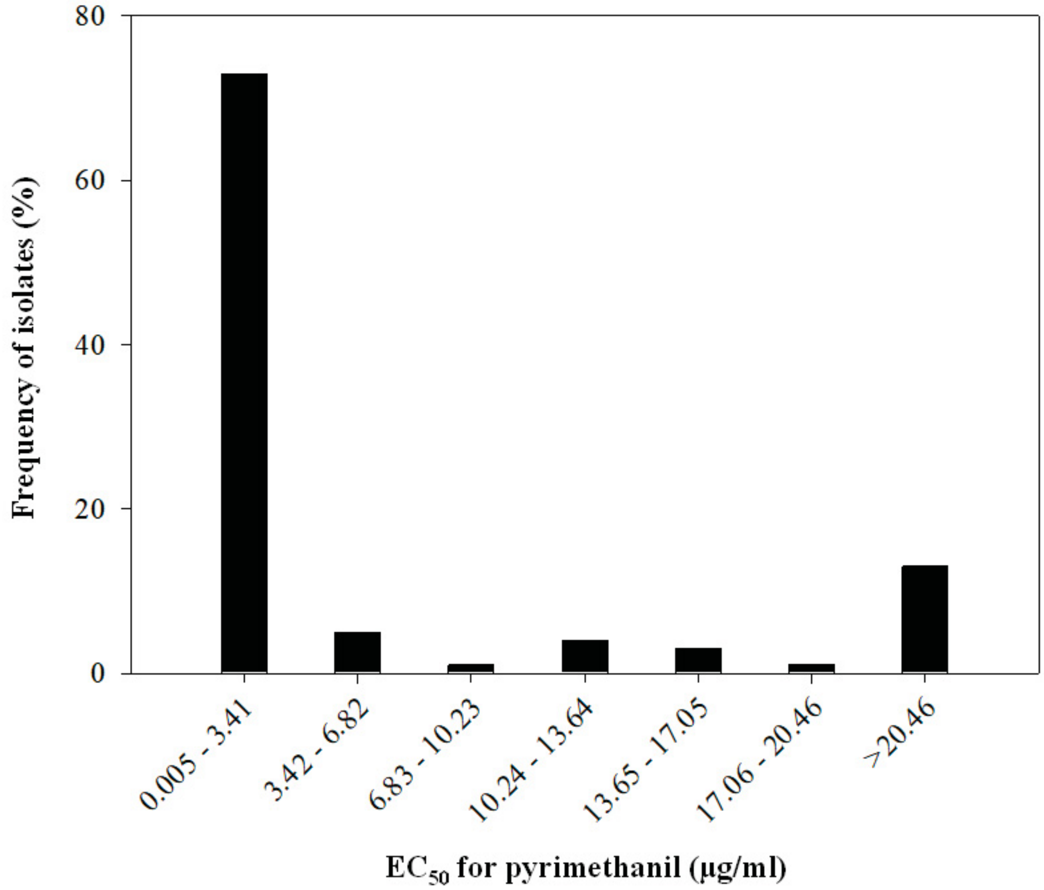

Fig. 2 Frequency distribution of EC_{50} values for pyrimethanil of 100 *Botrytis cinerea* isolates collected in different regions of the state of Paraná, Brazil. Individual EC_{50} values were grouped into class intervals of 3.4 µg/ml.

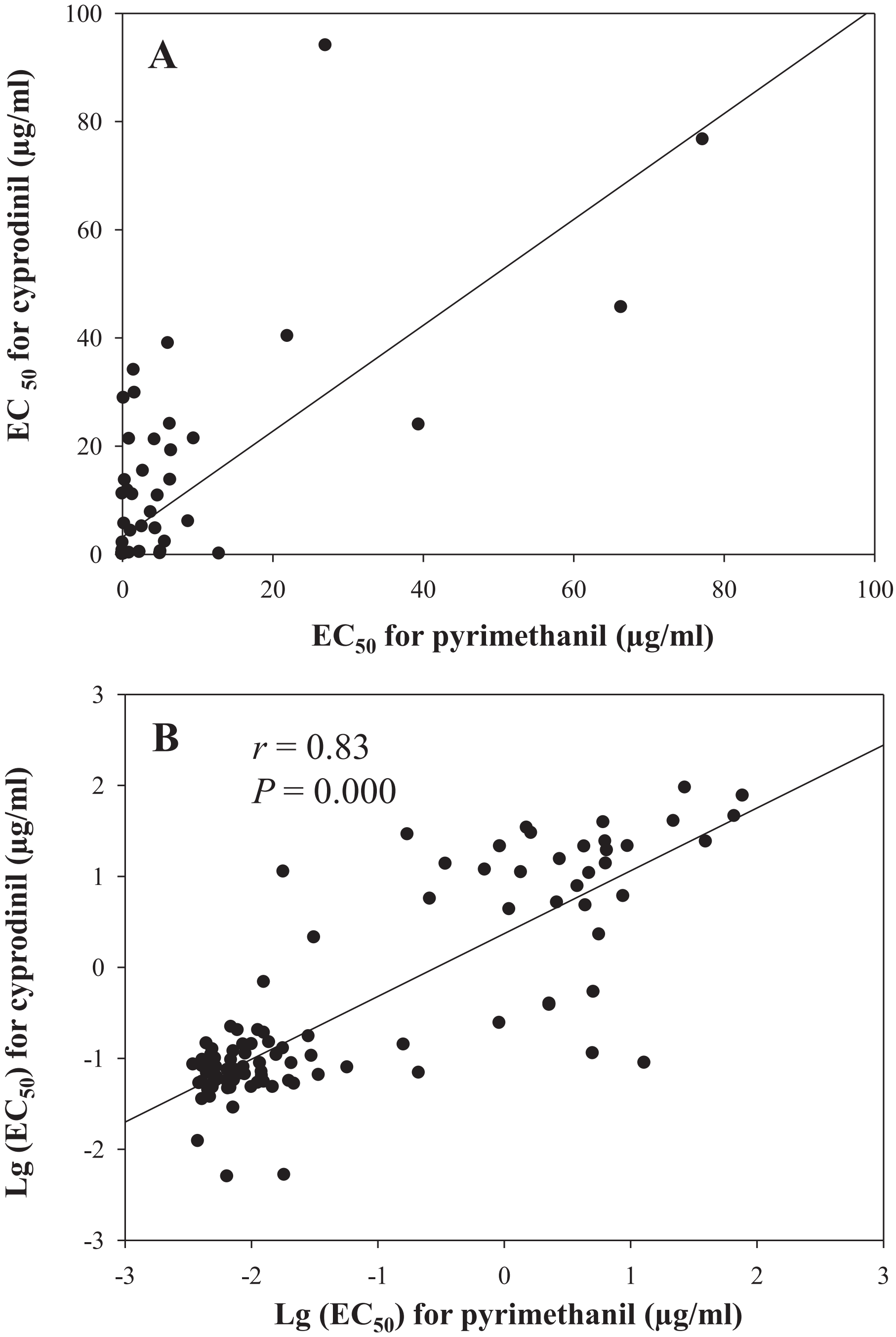

Fig. 3 Cross-resistance correlation analysis between pyrimethanil and cyprodinil in *Botrytis cinerea* isolates from strawberry. A - EC_{50} values and B – EC_{50} values in log.

Table 1 EC_{50} for pyrimetanil by region of Paraná state.

Region	Number of isolates	Average EC_{50} (µg/ml)	Range (µg/ml)
Southwest	2	3.12	0.203 – 6.04
Southeast	8	10.08	0.053 – 45.64
Metropolitan region of Curitiba	65	4.49	0.005 – 76.68
North	25	10.95	0.012 – 94.07

Table 2 Gray mold incidence in strawberry fruits following preventive and curative treatment with the fungicides pyrimethanil, cyprodinil and fludioxonil.

Treatment	Disease incidence(%)	
	Preventive	Curative
Control	81.25 Aa*	93.75 Aa
Pyrimethanil	12.50 Bb**	27.08 Ab
Cyprodinil	10.42 Bb	26.16 Ab
Fludioxonil	0.00 Ac	2.08 Ac

* Means followed by the same letters, lowercase in the column and uppercase in the row do not differ, respectively, by the ANOVA and Tukey tests at 5% probability.

** The data were transformed into $(x+1)^{1/3}$ for analysis, however the data are presented in the original scale

5 CHAPTER 3: Incidence of *Botrytis cinerea* in strawberry transplants imported into Brazil and sensitivity of isolates to fungicides[3]

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Abstract. Strawberry growers in Brazil mainly use imported transplants, due to their higher quality in terms of plant health and productivity. However, there is a risk of entry of new *Botrytis* species and/or isolates resistant to different fungicides. In this work, the incidence of *Botrytis* in imported transplants was investigated, and the species found

[3] Prepared in accordance with the standards of European Journal of Plant Pathology

identified. Following this survey, the sensitivity of these isolates to the fungicides procymidone, fludioxonil, iprodione, cyprodinil, pyrimethanil, boscalid and fluazinam was evaluated and the pathogenicity in fruit was confirmed. The average incidence of *Botrytis* spp. in transplants imported from Chile, Argentina, and Spain was 43.5%. A total of 79 isolates were identified molecularly as *B. cinerea* and the pathogenicity in fruit was confirmed with a subsample of 14 isolates. To evaluate sensitivity to fungicides, the following discriminatory doses method were used: procymidone (P) (10 µg/mL), fludioxonil (F) (0.5 µg/mL), iprodione (I) (10 µg/mL), cyprodinil (C) (10 µg/mL), pyrimethanil (PY) (10 µg/mL), boscalid (B) (50 µg/mL) and fluazinam (FL) (1 µg/mL). As a result, 24 resistance phenotypes were identified and the most frequent was the phenotype with resistance to 3 fungicides (C-PY-B). The isolates with low sensitivity to cyprodinil and pyrimethanil fungicides, which are not yet widely used in Brazil, represent a risk for production and this fact should be considered in disease management.
Keywords: Anilinopyrimidines; chemical control; dicarboximides; gray mold; phenylpyrrole.

Introduction

The production of transplants is of great importance in the strawberry production chain, as they are usually replaced every year for stronger plants and for the reduction of diseases and pests (Antunes et al., 2015). Therefore, is essential to have good quality transplants for good yield and healthy fruit. There are some essential prerequisites used to determine the transplant quality, such as healthy leaf area with two to three leaves; no symptoms of pests and diseases; transplant size of approximately 15 cm (Rufato et al., 2023). Although Brazil has been advancing in the production of transplants, there are still phytosanitary problems, so growers end up opting for imported transplants (Antunes and Cocco, 2012). In southern Brazil, the transplants used by strawberry growers are mainly from Argentine Patagonia and Chilean Patagonia (Costa et al., 2018). Imported transplants have a higher cost due to the phytosanitary inspections carried out to prevent new diseases from entering the national territory and costly transportation (Nunes et al., 2018; Schmitt et al., 2016;). As the production of bare-root transplants in Argentina and Chile occurs during the summer, when the average temperatures are 20°C, with low rainfall, it results in a low incidence of diseases (Rufato et al., 2023).

One of the main diseases that cause losses to strawberry growers is gray mold, caused by *Botrytis cinerea,* a pathogen capable of infecting various parts of the plant (Williamson et al., 2007). The fungus can remain latent for a long period, and latent infection offers *B. cinerea* an essential mechanism for survival. Within the living epidermal cell, the pathogen is protected from adverse weather conditions, interference from other pathogens and the effects of protective fungicides, lasting up to 8 months in leaves (Braun and Sutton, 1988). Thus, the transplants produced can be an important source of primary inoculum and transport the pathogen through latent infections to commercial strawberry fields, as has already been observed in the USA (Oliveira et al., 2017). Different subpopulations of *Botrytis* spp. may be selected in transplant nurseries under different fungicide spraying conditions (Amiri et al., 2018).

The genus *Botrytis* has more than 30 phytopathogenic species (Fillinger & Elad, 2015). Among these, *B. cinerea, B. fragariae* (Rupp et al. 2017), *B. caroliniana* (Li et al., 2012), *B. mali* (Dowling & Schnabel, 2017), *B. pseudocinerea* (Plesken et al., 2015) and *Botrytis* Group S (Leroch et al., 2013) are capable of infecting strawberry plants. In Brazil, *B. cinerea* is the species reported so far infecting strawberries (Lopes et al., 2017; Maia et al., 2021). Even though in countries from which Brazil has imported transplants there are no reports of these other species infecting strawberries (Farr & Rossman, 2021), *B. pseudocinerea* has been reported causing gray mold in vineyards in Spain (Acosta Morel et al., 2018). In Chile, the presence of *B. pseudocinerea* in peonies was reported (Muñoz et al., 2016). This species has also been reported causing gray mold on strawberries in Germany (Leroch et al., 2013; Plesken et al., 2015).

In addition to the entry of new species when importing transplants, we have the possibility of entry of isolates with resistance to the main fungicides used in the country. In the United States, isolates of *B. cinerea* from strawberry transplants showed, for the most part, resistance to 4 fungicides used in the management of strawberry diseases (Oliveira et al., 2017). Studies on the incidence of *Botrytis* in imported strawberry transplants have not yet been performed in Brazil. In a study with peach fruit imported from Spain, Chile, United States and Argentina, isolates of *Monilinia* spp. with resistance to the fungicides azoxystrobin, tebuconazole, iprodione and thiophanate methyl, widely used to control brown rot in Brazil were found (Pereira et al., 2018), which may pose a risk to local production.

In strawberry, the lack of sensitivity of isolates from transplants can directly interfere with disease management. If these isolates are as competitive as field isolates and adapt to adverse conditions, the efficacy of these fungicides may be impaired. This shows the importance of monitoring the strawberry transplants that are being imported for the entry of possible new species of *Botrytis* and isolates resistant to the fungicides used in the country, especially those that still perform well against gray mold in the country. Therefore, the objectives of this study were to: i) evaluate the incidence of *Botrytis* from imported transplants, ii) identify the *Botrytis* species of the isolates from transplants and iii) evaluate the sensitivity of these isolates to the fungicides procymidone, fludioxonil, iprodione, cyprodinil, pyrimethanil, boscalid and fluazinam.

Material and methods

Assessment of the incidence of *Botrytis* spp., isolate obtention and confirmation of pathogenicity

In 2021 and 2022, transplants imported from Chile, Argentina and Spain were purchased to detect *Botrytis* spp. (Fig.1). The cultivars used were based on the availability of importers, namely: Albion, Aromas, Camino Real, Fronteras, Monterey, Portola, and San Andreas. Fifty transplants per cultivar/country were obtained, totaling 1000 transplants.

The transplants were surface disinfected with 0.5% sodium hypochlorite for 2 min and then washed twice with sterile distilled water. They were then frozen overnight at -18 °C in a freezer to accelerate tissue death and then incubated inside a gerbox over a wire mesh, with distilled water at the bottom to maintain high humidity. The boxes were incubated for 7 days at 22 °C in BOD (Oliveira et al., 2017). The incidence of *Botrytis* spp. in transplants/cultivar/country was performed by observation under a stereoscopic microscope. Isolations were performed by placing the pathogen structures onto plates containing PDA medium, resulting in 79 *Botrytis* spp isolates. In order not to lose their characteristics after successive replications, all isolates were stored in silica gel using filter paper disks and stored in 30% glycerol, both at -18ºC in a freezer. The collection of isolates was deposited at the Laboratory of Epidemiology for Integrated Management of Diseases (LEMID) at the Federal University of Paraná.

For the evaluation of pathogenicity, 14 isolates from the collection were selected, coming from Argentina (1), Chile (8) and Spain (5). For each isolate, eight replicates were used. Each replicate consisted of three strawberry fruit inside a plastic pot of 15 cm in diameter and 4.5 cm in height with the presence of cotton and filter paper sterilized and moistened with distilled water. The fruit used were Albion cultivar at the green to red maturation stage, without fungicide residues. These fruits were disinfected with 0.5% sodium hypochlorite for 2 min, washed three times in distilled water and dried at room temperature. The fruit were placed inside disinfected plastic containers and 50 µL of suspension at 1×10^5 conidia/mL were deposited on their surfaces in a previously determined location on each uninjured fruit. The control treatment consisted of applying 50 µL of distilled water to uninjured fruit. The fruit were incubated at 22ºC with a 12 h photoperiod and evaluated at intervals of 24 hours up to 5 days for the presence of symptoms and signs of the pathogen.

Identification of the *Botrytis* species of the collected isolates

All 79 isolates obtained were molecularly identified. For this, the DNA of each isolate was extracted using the CTAB protocol, with some modifications (Doyle and Doyle 1987; Pereira et al. 2019). Species identification was performed using PCR, using the forward primers G3PDH-F1 and G3PDH-F2 together with the reverse G3PD-R to identify *B. caroliniana* and *B. cinerea*, respectively (Li et al. 2012). The reaction was run in a total reaction volume of 12.5 µL, consisting of 6.25 µL of PCR Master Mix 2x (Promega), 10 µM of each primer and 1.5 µL of DNA. Amplification consisted of an initial denaturation at 94°C for 3 min followed by 32 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were analyzed on a 1% agarose gel in 0.5X TBE buffer, stained with gelRed (Biotium) and photo documented on an ultraviolet transilluminator.

Sensitivity to fungicides – Discriminatory dose

The sensitivities of *Botrytis* spp. to the fungicides procymidone (Sumilex 500 WP®, Sumitomo), fludioxonil (Maxim®, Syngenta), iprodione (Rovral SC®, Basf), cyprodinil (Unix 750 WG®, Syngenta), pyrimethanil (Mythos®, Bayer), boscalid (Cantus ®, Basf) and fluazinam (Frowncide 500 SC®, Ihara). Stock solutions of fungicides were prepared with sterile distilled water. The sensitivity of the 79 *Botrytis* isolates to fungicides was evaluated using discriminatory dose by mycelial growth or spore germination inhibition assays depending on the fungicide.

For procymidone, iprodione, fludioxonil, cyprodinil and pyrimethanil fungicides, the discriminatory dose was determined by mycelial growth inhibition assays. The doses used to discriminate the sensitivity of the isolates were: I) 10 µg/mL for procymidone and iprodione, II) 0.5 µg/mL for fludioxonil and III) 10 µg/mL for cyprodinil and pyrimethanil. Commercial fungicides were diluted with sterilized distilled water to obtain stock solutions, which were later added to autoclaved potato-dextrose-agar (PDA) medium and cooled down to 60°C. The medium was added to 90 mm Petri dishes and 5 mm diameter mycelium discs, obtained from seven-day-old colonies, were deposited on the surface of the culture medium. For cyprodinil and pyrimethanil, sensitivity was evaluated in mycelial growth inhibition assay on an agar medium based on L-asparagine-based agar medium (ASP-agar) (Hilber and Schuepp, 1996). The control treatment consisted of placing the mycelium discs from the isolates on PDA or ASP-agar medium without fungicide. A total of 2 plates were used per isolate/discriminating dose. Plates were incubated at 22°C with a 12-hour photoperiod for two days for PDA medium and 5 days for ASP-agar. Colony diameter was obtained by averaging two perpendicular measurements of the colony using a digital caliper.

For the fungicides boscalid and fluazinam, which act on germination, the discriminatory dose was determined by spore germination. The doses used were I) 50 µg/mL for boscalid and II) 1 µg/mL for fluazinam. The stock solutions were added to the autoclaved water-agar (WA) medium and kept at 60°C until the discriminatory doses were obtained. The medium with fungicide was added to 90 mm diameter Petri dishes. Mycelium discs 5 mm in diameter from the isolates were transferred to canned peaches for inoculum production. Sporulation on the surface of canned fruit was used to produce spore suspensions. Aliquots of 100 µl of the suspension containing 10^5 conidia/ml of each isolate were added to the surface of the culture media and spread with the aid of a Drigalski loop. Plates were incubated at 22°C for 14 h in the dark. After this period, germination was stopped by adding lactophenol with Amann blue dye to the plates. A total of 2 plates were used per isolate/discriminatory dose. One hundred conidia per plate were evaluated. Conidia were considered germinated when they had germ tubes at least twice their size. The mycelial growth inhibition percentage ratio was calculated using the formula %ICM= [(C-T) /C] × 100, where C refers to the diameter of the control and T to the average diameter of the treatment with fungicide. The inhibition of germination of conidia was calculated using the formula %IGC =[(C-T)-C]X100, where C is the number of conidia germinated in the control and T the number of conidia germinated in the treatment. The experiments were performed twice. The phenotypic classification of isolates was performed according to Table 1. Potential cross-resistance between pyrimethanil and cyprodinil was evaluated using the mycelial growth inhibition value to determine the phenotypes for cyprodinil and pyrimethanil, for the same 79 isolates, at a dose of 10 µg/ml. Cross-resistance analysis was performed by calculating Pearson's correlation coefficient (r).

Results

Incidence, identification of *Botrytis* spp. in transplants and pathogenicity of isolates

The average incidence of *B. cinerea* in transplants was 43.5% and a total of 79 isolates were obtained. The highest incidences were in the San Andreas and Monterey cultivars imported from Spain in 2022, with 88 and 90% incidence, respectively (Table 2). The cultivar from Chile that presented the highest incidence was Albion in 2022, with 68% of the transplants with the presence of *B. cinerea,* and the San Andreas cultivar from Argentina that presented an incidence of 64% (Table 2). Fragment analysis of the Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene showed the presence of a fragment of 238 base pairs in all isolates, identifying them as *Botrytis cinerea*. The 14 selected isolates showed pathogenicity to the fruit, with the presence of symptoms and signs being observed from 36 hours after inoculation. On the fifth day, the final incidence was above 75% for all isolates.

Fungicide Sensitivity – Discriminatory Dose and Cross-Resistance

Discriminatory doses allowed to distinguish different *B. cinerea* sensitivity patterns to fungicides. For the seven fungicides used in the sensitivity tests, it was possible to observe 24 different phenotypes. Of the total of 79 isolates, only two isolates were sensitive to all fungicides (Fig. 2). On the other hand, two isolates were resistant to 6 of the 7 evaluated fungicides. The most frequent phenotype was C-PY-B, resistant to 3 fungicides, with a frequency of 24.1% (Fig. 2). The percentage of isolates sensitive to procymidone, fludioxonil, iprodione, cyprodinil, pyrimethanil, boscalid, and fluazinam was 86.1; 89.9; 81; 21.5; 21.5; 21.5; 38.0 and 94.9%, respectively (Fig.3A). No significant differences were observed between the sensitivity frequencies of isolates from Chile and Argentina compared to isolates from Spain (Fig. 3B and 3C). The correlation between the percent mycelial growth inhibition of pyrimethanil and cyprodinil was significantly positive ($r = 0.76$, $P = 0.000$).

Discussion

Our study shows that transplants are an important source of primary inoculum for gray mold due to their infection by *B. cinerea*. Isolates are being introduced every year from different sources around the world, representing a risk of entry of new species. In this study, only *B. cinerea* was detected, but the isolates showed reduced sensitivity to multiple fungicides, including fungicides recently introduced to control the disease in Brazil, to which our local population is still highly sensitive.

In Brazil, no new species of *Botrytis* causing gray mold on strawberries have yet been found (Lopes et al., 2017; Maia et al., 2021). However, one of the ways that new species can enter the country is through transplants. Our study showed that, so far, only *B. cinerea* has been introduced with imported transplants. This is important to report since in a study carried out in the USA a small frequency of *Botrytis* group S isolates was found coming from nurseries in the USA and Canada (Amiri et al., 2018).

A high incidence of *B. cinerea* was observed entering the country with the transplants, and that opens the possibility of the entry of isolates with reduced sensitivity to the main fungicides used in Brazil since several fungicides sprayed by the growers can also be used in nurseries to produce healthy transplants (Oliveira et al., 2017). In Spain, the fungicides registered for use on strawberry that were tested in this study were fludioxonil, pyrimethanil and the mixture fludioxonil + cyprodinil (MAPA, 2023). In Argentina and Chile, the fungicides procymidone, iprodione, and the mixtures fludioxonil+cyprodinil and boscalid+pyraclostrobin are registered for strawberry, with pyrimethanil registered only in Chile (CASAFE, 2023; SAG, 2023) (Table 3).

Historically, there are many reports of resistance of *B. cinerea* isolates to several fungicides in the world: azoxystrobin (Ishii et al., 2009), boscalid (Bardas et al., 2010; Cui et al., 2021), procymidone (Liu et al., 2010; al., 2016), iprodione (Cosseboom and

Hu, 2021), cyprodinil (Cosseboom and Hu, 2021) and fludioxonil (Sang et al., 2018; Zhou et al., 2020; Dowling et al., 2021). In Brazil, resistant isolates have already been found for azoxystrobin, boscalid, iprodione, procymidone, methyl thiophanate, pyrimethanil and cyprodinil (Lopes et al., 2017; Baggio et al., 2018; Maia et al., 2021; 2023). The frequency of 86.1% of isolates sensitive to procymidone was higher in relation to the previous study carried out with isolates of *B. cinerea* from the state of Paraná, where 57% of the isolates were classified as susceptible (Maia et al., 2021). The same occurred with iprodione, where the value of 81% of isolated patients was higher than the 56% found by Maia et al. 2021. Fluazinam showed 61.9% efficacy in controlling gray mold in the state of Paraná (Maia et al., 2021). Therefore, the frequency of 96.3% of fluazinam-sensitive isolates is a good indicator, since the entry of *B. cinerea* isolates through the transplants does not represent a great risk for the efficacy of this fungicide. Fluazinam is not registered for use on strawberries in Argentina, Chile, and Spain, which explains the low frequency of resistant isolates (MAPA, 2023). Fludioxonil was recently registered in Brazil in a mixture with cyprodinil for the management of strawberry diseases and proved to be the most effective in controlling gray mold (unpublished data). Thus, it is important to avoid the entry of isolates with reduced sensitivity to this fungicide. In this study, 10.1% of the isolates showed resistance to fludioxonil. Although this is not a high value, the alert remains as in Spain, Argentina, and Chile this fungicide is already registered and can be used in the production of transplants. In Spain, in addition to the fludioxonil+cyprodinil mixture, fludioxonil is also registered as a solo product for use in strawberries (MAPA, 2023). Therefore, fungicides containing fludioxonil in their composition should be avoided in the control of diseases in transplant production nurseries.

For boscalid, cyprodinil and pyrimethanil fungicides, most isolates showed low sensitivity and 59.3, 78.5 and 78.5% of isolates were classified as resistant, respectively. The frequency of boscalid-resistant isolates in this study was higher than that found in a study with isolates collected in the state of Paraná, where the frequency of boscalid-resistant isolates was 45.3% (Maia et al., 2021). This fungicide was found to be highly effective in preventing gray mold in a previous study in Brazil, therefore the presence of resistant isolates is alarming as it can reduce the effectiveness of this product (Unpublished data). In another study also carried out in Brazil with isolates of *B. cinerea* from strawberry, only 12.5% of the isolates were classified as highly resistant to pyrimethanil (Baggio et al., 2018). This shows that within the country the frequency of pyrimethanil-resistant isolates is still low, contrary to what occurs in the rest of the world, where this fungicide has been used for much longer and pyrimethanil-resistant isolates are frequently found (Myresiotis et al., 2007, Zhao et al., 2010; Korolev et al., 2011). Furthermore, pyrimethanil is registered for use on strawberries in Chile and Spain with a higher recommended dose than that used in Brazil (SAG, 2023; MAPA, 2023; AGROFIT, 2023). Cyprodinil was registered for cultivation in Brazil only in 2019 in a mixture with fludioxonil and demonstrated an efficacy of 85.7% in the control of gray mold in strawberry, being an important fungicide in the management of strawberry diseases (unpublished data). Pyrimethanil and cyprodinil have similar chemical structures because they belong to the same chemical group, anilinopyrimidines, and cross-resistance may occur between the two fungicides (Leroux et al., 1999). When evaluating the cross-resistance between cyprodinil and pyrimethanil, we observed a positive correlation of 0.76. Positive correlations have already been found in Brazil and Greece, with values of 0.82 and 0.71, respectively (unpublished data; Myresiotis et al., 2006). This reinforces the risk of selecting isolates resistant to both fungicides.

The entrance of fungicide-resistant isolates of *B. cinerea* is concerning, as it may interfere with the management of the disease in the future, especially if these isolates can adapt and compete with the other isolates. In fruits imported from Chile, USA, and Argentina, many *Monilinia* isolates were resistant to iprodione, and there are no reports of isolates with resistance to this fungicide in Brazil, which could impair disease control in the country (Pereira et al., 2018). In the USA, a study showed the first indication that fungicide-resistant populations of *B. cinerea* were introduced into strawberry fields through transplants (Oliveira et al., 2017; Amiri et al., 2018).

In our study, resistance to up to 6 fungicides was observed, the most frequent phenotype being C-PY-B resistant to three fungicides, with a frequency of 24.1%. In the USA, the classification of isolates from transplants showed that the phenotype resistant to 3 fungicides was the most observed, with 42.1%, and isolates with resistance to 6 fungicides found simultaneously (Oliveira et al., 2017). As Oliveira et al. 2017, we also found the presence of *B. cinerea* in transplants and proved that these populations are resistant to one or several fungicides simultaneously. Competitiveness and adaptability tests are still needed to measure the importance of the entry of these isolates in Brazil since it is already known in the literature that isolates with multiple resistance may not compete equally with sensitive isolates. The evolution of resistance to fungicides in fungal populations depends on the fitness of the resistant population, having important implications in the management of the disease (Bardas et al., 2008).

Strawberry growers in Brazil are receiving transplants already infected with *B. cinerea* isolates resistant to several fungicides, some of which do not yet represent a risk of control failure in the field in the country. This discovery is very relevant and should alert nurseries and strawberry growers to adopt new strategies in the management of strawberry diseases. One of the ways that strawberry growers can reduce the primary inoculum of *B. cinerea* in the transplants is by immersing the transplants in known effective fungicides before planting or spraying immediately after planting (Oliveira et al., 2017). When receiving the transplants, the growers are not instructed to carry out any treatment before planting, and some end up immersing the transplants in fungicides such as thiophanate methyl and azoxystrobin. These fungicides already have low efficacy against gray mold in Brazil and should not be used to control the disease (Baggio et al., 2018; Maia et al., 2021). The use of heat treatment at 44°C for 4h has already been shown to be highly effective in reducing *B. cinerea* inoculum in strawberry transplants (Zuniga et al., 2023).

Our results show that it is important to continue monitoring imported transplants both for new species and possibly new diseases and for the sensitivity of these isolates to the fungicides used in the country. It is also important to check the adaptability and competitiveness of isolates with resistance to various fungicides. If they are competitive and adapt to different conditions, the problem of entry of these isolates can be aggravated. An important point to consider is that if national transplants are used, the problem of introducing resistant isolates into new areas can also occur as with the use of imported transplants, depending on the fungicides used in the nurseries. Therefore, monitoring the sensitivity of *B. cinerea* to fungicides in transplant production nurseries can help in the management of strawberry diseases, preventing isolates from being selected for resistance to fungicides that present high control efficacy, and subsequently being disseminated through transplants.

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethics declarations

All authors declare that this material has not been published in whole or in part elsewhere; the manuscript is not currently being considered for publication in another journal; all authors have been personally and actively involved in substantive work leading to the manuscript and will hold themselves jointly and individually responsible for its content. We declare that our manuscript complies with all ethical rules applicable for this journal and that there are no potential conflicts of interest or even research involving human participants and/or animals.

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Table 1 Information on fungicides and methodologies used to assess *Botrytis cinerea* sensitivity using discriminatory doses.

Active ingredient	Discriminatory dose	Classification	Reference
Boscalid	50 µg/ml	R- ICG <80%	Fernandez-Ortuño et al., 2016
Cyprodinil	10 µg/ml	R- IMG <80%	Autor
Fluazinam	1 µg/ml	R- ICG <50%	Maia et al., 2021
Fludioxonil	0,5 µg/ml	R- IMG <80%	Fernandez-Ortuño et al., 2014 modified
Iprodione	10 µg/ml	R- IMG <80%	Fernandez-Ortuño et al., 2014 modified
Pyrimethanil	10 µg/ml	R- IMG <80%	Autor
Procymidone	10 µg/ml	R- IMG <80%	Fernandez-Ortuño et al., 2014 modified

R- resistant, IMG - inhibition of mycelial growth and ICG- inhibition of conidial germination

Table 2 Incidence of *Botrytis cinerea* in transplants national and imported from Chile, Argentina, and Spain

Cultivar	Country	Nº of transplants	Year	Incidence of *B. cinerea* (%)	Nº of isolates
Camino real	Chile	50	2021	56	3
Fronteiras	Chile	50	2021	28	2
Albion	Chile	50	2021	22	0
San Andreas	Chile	50	2021	28	7
Monterey	Chile	50	2021	58	6
San Andreas	Spain	50	2021	20	10
Albion	Spain	50	2021	46	10
Monterey	Spain	50	2021	28	8
San Andreas	Argentina	50	2021	64	3
Aromas	Chile	50	2021	36	0
Portola	Chile	50	2021	10	0
Albion	Spain	50	2022	78	5
Monterey	Spain	50	2022	90	11
San Andreas	Spain	50	2022	88	6
Aromas	Chile	50	2022	12	0
Camino real	Chile	50	2022	24	4
Portola	Chile	50	2022	0	0
Monterey	Chile	50	2022	52	1
San Andreas	Chile	50	2022	62	2
Albion	Chile	50	2022	68	1
Total		1000			79

Table 3 Fungicides registered in Argentina, Brazil, Chile, and Spain for use on strawberry

Registered active ingredients for strawberry	Separate active ingredient	Amount of active ingredient recommended for culture			
		Argentina	Brazil	Chile	Spain
Procymidone	-	500 g/ha	500 g/ha	375 g/ha	-
Fludioxonil	-	-	-	-	250 g/ha
Iprodione	-	1000 g/ha	750 g/ha	750-1000 g/ha	-
Pyrimethanil	-	-	360 g/ha	800 g/ha	600-800 g/ha
Boscalid	-	-	400 g/ha	-	-
Fluazinam	-	-	500 g/ha	-	-
Fludioxonil+Cyprodinil	Fludioxonil	125-150 g/ha	200-250 g/ha	200-300 g/ha	150-250 g/ha
	Cyprodinil	187.5-225g/ha	300-375 g/ha	300-450 g/ha	225-375 g/ha
Boscalid+ Pyraclostrobin	Boscalid	189 g/ha	-	60.5- 90.7 g/ha	-
	Pyraclostrobin	96 g/ha	-	30.7-46 g/ha	-

- doses not found for strawberry applications in the searched sources.

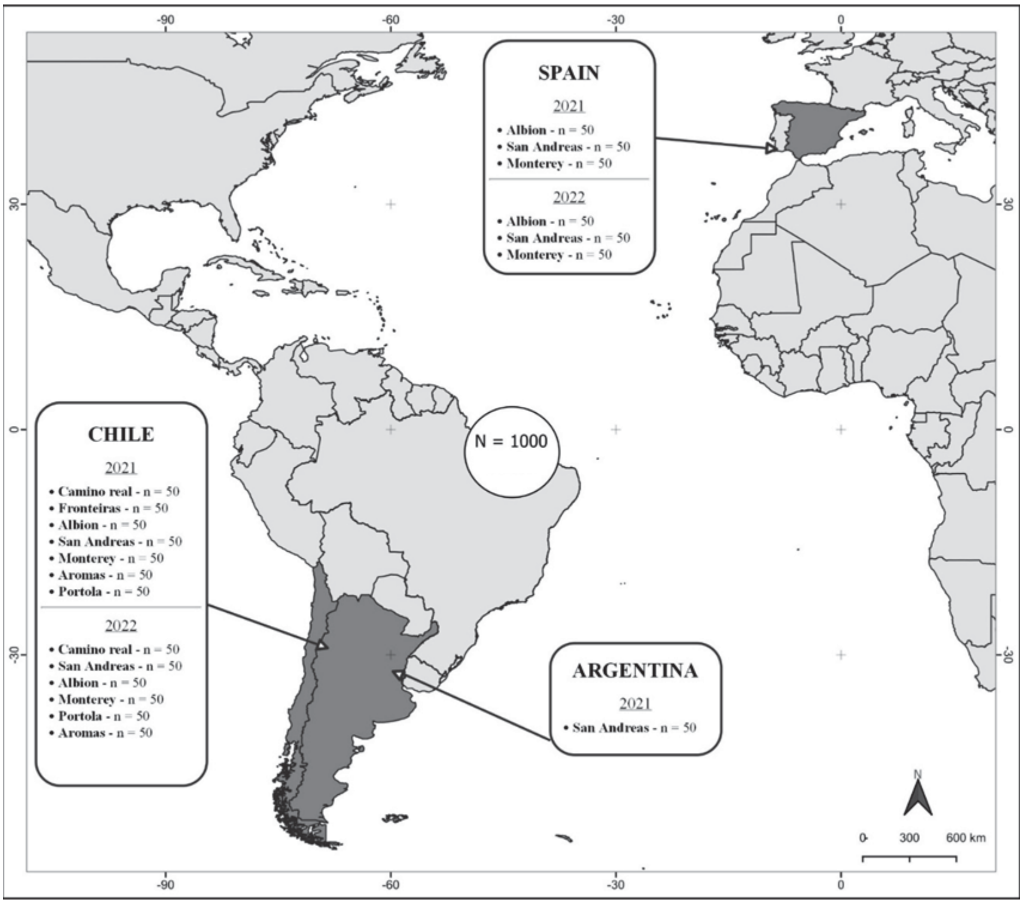

Fig.1 Origin, year of importation and cultivars of the transplants that were used in this study

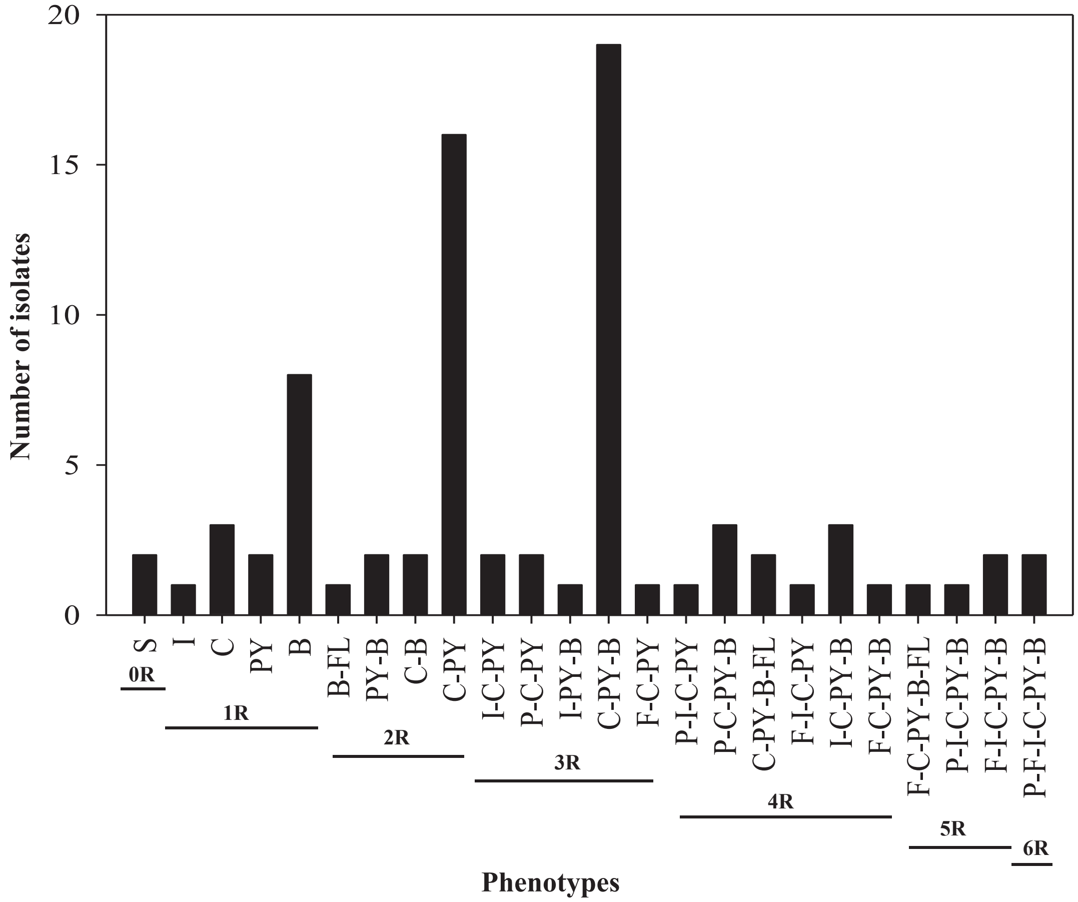

Fig. 2 Phenotypic classification of the sensitivity of *Botrytis cinerea* isolates to the fungicides procymidone, fludioxonil, iprodione, cyprodinil, pyrimethanil, boscalid and fluazinam. S- Sensitive, P- procymidone, F- fludioxonil, I- iprodione, C- cyprodinil, PY- pyrimethanil, B- boscalid, FL- fluazinam. 0R- without resistant to the fungicide,1R-resistant to a fungicide, 2R-resistant to two fungicides, 3R-resistant to three fungicides, 4R-resistant to four fungicides, 5R-resistant to five fungicides and 6R-resistant to six fungicides.

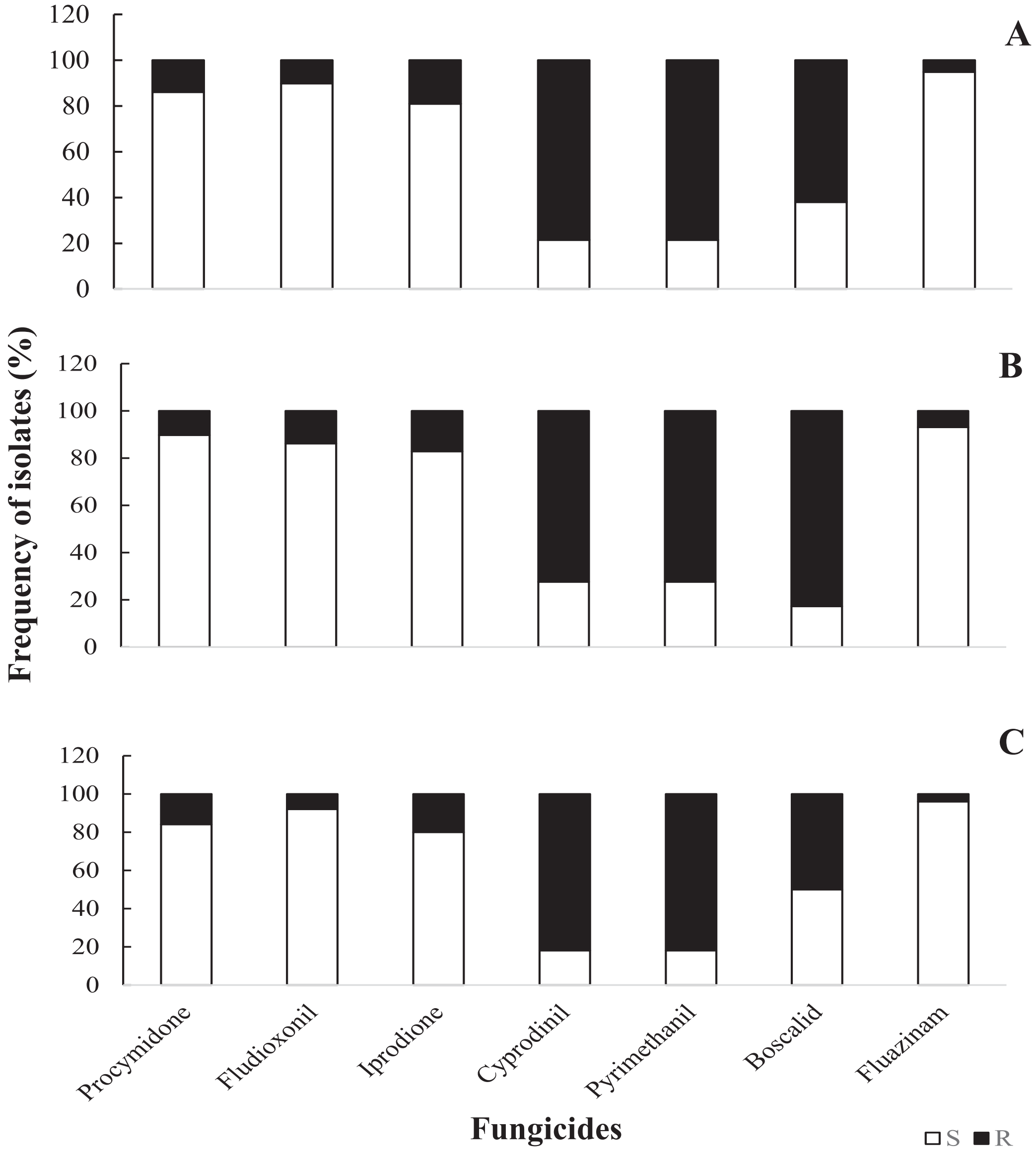

Fig. 3 Characterization of *Botrytis cinerea* isolates sensitivity to procymidone, fludioxonil, iprodione, cyprodinil, pyrimethanil, boscalid and fluazinam. Frequency of sensitive (S) (white) and resistant (R) (black). A- All isolates, B- Chile and Argentina isolates and C- Spain isolates.

6 FINAL CONSIDERATIONS

In this study, we found fungicides that can be incorporated into the management of strawberry gray mold in Brazil. In addition, the importance of transplants as a source of primary inoculum for *B. cinerea* and the entry of isolates resistant to multiple fungicides in Brazil was shown.

The most recently registered fungicide for strawberry, the mixture fludioxonil + cyprodinil, can play an important role in disease management, but the risk of resistance must be considered to prolong the use of this fungicide. For this, future monitoring needs to continue our work and anti-resistance measures will have to be incorporated. This is also recommended for pyrimethanil, which still has high control efficacy but has a medium risk of resistance. Attention to the frequency of application and the rotation of the fungicides used should be paid.

In the study carried out with transplants, we collected important information regarding the entry of *B. cinerea* isolates into the country. They were often found in imported transplants and had low sensitivity to several fungicides used in Brazil. This was the first study of imported strawberry transplants in Brazil and opens several questions to be answered in future studies, as the adaptability and competitiveness of these isolates with isolates from commercial strawberry fields. In addition, treatments must be evaluated to avoid planting transplants with latent *B. cinerea*.

All the results shown in this study were aimed at helping strawberry growers in Brazil to have access to better management of gray mold, indicating fungicides with better efficacy than those more frequently applied, alerting that imported transplants are a potential source of inoculum, and recommending the best transplants treatment before planting.

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