GABRIEL MODESTO BEGHELLI

COMPATIBILITY OF A PREDATOR AND AN ENTOMOPATHOGENIC FUNGUS OF THE ASIAN CITRUS PSYLLID

Dissertation submitted to the Entomology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

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“Deus não poderia me inspirar desejos irrealizáveis”.
Santa Teresa de Lisieux

ABSTRACT

BEGHELLI, Gabriel Modesto, M.Sc., Universidade Federal de Viçosa, July, 2024. **Compatibility of a predator and an entomopathogenic fungus of Asian citrus psyllid**. Adviser: Angelo Pallini. Co-advisers: Arne Janssen and Madelaine Venzon.

The Asian citrus psyllid *Diaphorina citri* Kuwayama (Hempitera: Pysllidae) is a major pest of citrus because it vectors bacteria associated with Huanglongbing, the most destructive citrus disease. This disease causes severe economic losses in citrus production worldwide. Attempts to prevent disease dissemination have mainly focused on use of pesticides to control the vector, but this did not prevent disease spread. Besides, the intensive use of pesticides reduces densities of natural enemies and favors the development of resistance in the psyllids, resulting in pest resurgence. Thus, other strategies are needed for control of *D. citri* and Huanglongbing. One promising method is biological control; several natural enemies of psyllids in citrus orchards have been identified, but of those, only entomopathogenic fungi are commercially available, for instance *Cordyceps fumosorosea* (Wize, 1904) (Hypocreales: Cordycipitaceae). Generalist predators show great potential in preventing psyllid invasions and reducing disease spread because they can be released preventively with the addition of alternative food. The predatory mite *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae) is such a generalist and showed promise in controlling *D. citri*. However, little is known about its compatibility with the entomopathogen. Recent studies have shown that predatory mites can vector fungal spores to pests. Hence, the concurrent use of predators and entomopathogens may enhance control of *D. citri*. However, those agents could also affect each other's actions negatively: entomopathogenic fungi can potentially infect predatory mites. Therefore, I studied the pathogenicity of *C. fumosorosea* for *A. herbicolus*. I found that the fungus had no detrimental effect on survival, development and reproduction of the predator, suggesting that both agents can be used together for the control of the psyllid. I also aimed to assess the effects of concurrent use of predator and pathogen on the population dynamics of *D. citri*. The combination of both agents did not reduce psyllid densities on plants better than the pathogen alone. However, the predators went extinct on all plants during the experiment, and its reason remains to be investigated. In conclusion, life history traits of *A. herbicolus* are not influenced by direct exposure to C. *fumosorosea* but it is unclear if the simultaneous use of both agents would be beneficial for biocontrol of Asian citrus psyllid.

Keywords: Citrus greening. Biological control. Generalist predator. Hypocrealean fungus**.**

RESUMO

BEGHELLI, Gabriel Modesto, M.Sc., Universidade Federal de Viçosa, julho, 2024. **Compatibilidade de um predador e um fungo entomopatogênico do psilídeo asiático dos citros**. Orientador: Angelo Pallini. Coorientadores: Arne Janssen e Madelaine Venzon.

O psilídeo asiático dos citros *Diaphorina citri* Kuwayama (Hempitera: Pysllidae) é uma praga importante dos citros porque é vetor de bactérias associadas ao Huanglongbing, a mais destrutiva doença cítrica. Esta doença causa graves prejuízos econômicos à citricultura em todo o mundo. As tentativas de prevenir a disseminação da doença centraram-se principalmente na utilização de pesticidas para controlar o vector, mas isto não impediu a propagação da doença. Além disso, o uso intensivo de pesticidas reduz as densidades de inimigos naturais e favorece o desenvolvimento de resistência em psilídeos, resultando no ressurgimento da praga. Por isso, outras estratégias são necessárias para o controle de *D. citri* e de Huanglongbing. Um método promissor é o controle biológico; vários inimigos naturais do psilídeo foram identificados em pomares de citros, mas destes, apenas fungos entomopatogênicos estão disponíveis comercialmente, por exemplo *Cordyceps fumosorosea* (Wize, 1904) (Hypocreales: Cordycipitaceae). Predadores generalistas apresentam grande potencial na prevenção de invasões de psilídeos e na redução da propagação da doença, pois podem ser liberados preventivamente com a adição de alimentos alternativos. O ácaro predador *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae) é generalista e mostrou-se promissor no controle de *D. citri*. Entretanto pouco se sabe sobre sua compatibilidade com o entomopatógeno. Estudos recentes mostraram que ácaros predadores podem transportar esporos de fungos para as pragas. Dessa forma, o uso simultâneo de predadores e entomopatógenos pode aumentar o controle de *D. citri*. Contudo, estes agentes podem afeter negativamente a ação um dos outros: fungos entomopatogênicos potencialmente infectam ácaros predadores. Portanto, estudei a patogenicidade de *C. fumosorosea* para *A. herbicolus*. Descobri que o fungo não tem efeito prejudicial na sobrevivência, desenvolvimento e reprodução do predador, sugerindo-se ambos agentes podem ser utilizados em conjunto para o controle do psilídeo. Também tive como objetivo avaliar os efeitos do uso simultâneo do predador e do patógeno na dinâmica populacional de *D. citri*. A combinação de ambos os agentes não foi melhor que o uso isolado do pátogeno em reduzir a densidades de psilídeos nas plantas. No entanto, os predadores foram extintos de todas as plantas durante o experimento, e a razão para isto ainda precisa ser investigada. Em conclusão, os atributos de história de vida de *A. herbicolus* não são influenciadas pela exposição direta a *C. fumosorosea*, mas permanece incerto se o uso simultâneo destes agentes seria benéfico para o controle biológico do psilídeo asiático dos citros.

Palavras-chave: Greening dos citros. Controle biológico. Predador generalista. Fungo da ordem Hypocreales.

SUMMARY

Chapter 1:

Chapter 2:

GENERAL INTRODUCTION

Through time, humans have employed different practices to control pests on crop plants (Hajek and Eilenberg 2018). During World Wars I and II, the chemical industry passed through a technological revolution that favored the large-scale production of synthetic pesticides (Hajek and Eilenberg 2018). Since then, the use of pesticides has increased and chemical control is currently the most used strategy to manage pests (Hajek and Eilenberg 2018). Nevertheless, the perception of pesticides has changed in the past decades (Coats 1994), motivated by serious problems caused by their use. They are harmful to non-target organisms, including the natural enemies of plant pests, and consequently cause pest resurgence and outbreaks of secondary pests (Zanardi et al. 2018; Janssen and van Rijn 2021). The continuous use of pesticides also results in the selection of pest resistance (Bras et al. 2022), pesticides are related to worldwide decline of pollinators (Goulson et al. 2015), and are harmful to human health and the environment (Pimentel 1996; Nicolopoulou-Stamati et al. 2016; Rani et al. 2021).

Therefore, environmental-friendly methods to control pests have gained importance as an alternative to pesticides. Is this sense, biological control is considered as an effective and sustainable approach of pest control (Brodeur et al. 2018). Biological control can be defined as the use of living agents to control pestilent organisms according to human benefit (Stenberg et al. 2021). Accordingly, biological control in agriculture is a management strategy in which pest species are held at low densities by the action of natural enemies (Huffaker 1985). Biological control dates from ancient history: the earliest record is from 304 a.D. in China, with the use of weaver ants to control citrus pests (van den Bosch et al. 1982; Huang and Yang 1987). Biological control is commonly divided in three types: classical, augmentative and conservation; but the definition of each type varies depending on literature (Bale et al. 2008). Besides, other categories are found in literature, for example neoclassical, inoculation and inundation biological control (Eilenberg et al. 2001). Some authors proposed terminology with four classes of biological control: natural, conservation, classical and augmentative (van Lenteren et al. 2018; Stenberg et al. 2021). The first two classes involve organisms that are resident in the ecosystem: natural biological control when control provided by resident agents does not depend on human intervention and conservation biological control when humans act in the ecosystem to increase control by these resident agents. The other two classes are related with addition of organisms to the ecosystem. Classical biological control is the addition of agents that are supposed to become permanently established, augmentative biological control concerns the release of organisms are temporarily established for pest control.

Most arthropod pests can be controlled with biocontrol agents (van Lenteren and Nicot 2020). There is a great diversity of natural enemies of arthropod pests. Among them are predators, pathogens and parasitoids (DeBach and Rosen 1991; Eilenberg et al. 2001). Despite this diversity, studies historically focused on a single enemy species that controlled a single pest species (Cardinale et al. 2003; Messelink et al. 2012). Notwithstanding, in agricultural food webs, multiple interactions occur among several natural enemies and herbivores, impacting biological control (Janssen et al. 1998). Interactions among multiple organisms are complex. Single natural enemies sometimes control a pest species better when another pest is present (Liu et al. 2006; Messelink et al. 2008, 2010), but biological control of one herbivore species may also be disrupted by the presence of other pests (Koss and Snyder 2005).

Diversity of enemies also plays an important role in the suppression of herbivores (Jonsson et al. 2017). Several researchers investigated whether enemy assemblages provide better pest control than single enemy species, showing that the effects of enemy diversity on biological control varied among studies (Rosenheim et al. 1995; Snyder and Ives 2001, 2003; Denoth et al. 2002; Cardinale et al. 2003; Griffin et al. 2013; Roubinet et al. 2015). In short, combinations of two biocontrol agents can lead to five results: synergism, independent additive, additive, non-additive and negative effects (Wraight 2003). An example is given to clarify the differences among these results. Suppose a biocontrol agent species reduces densities of a pest with 40%, while another agent species reduces densities of this pest by 30%. Synergism occur when the combined action of agents reduces pest densities to a level higher than the summed effect of each agent alone (reduction of pest densities higher than 70% in the proposed example). Independent additivity is when concurrent agents use reduce pest densities equal to the summed effect of each agent alone (70% of reduction on pest densities). Additive effects occur when the combined action of agents results in a level of control higher than that provided by the most effective agent alone, but lower than the summed effect of each agent alone (pest control higher than 40% and lower than 70%). Non-additive, or neutral, effects means that the control provided by natural enemies together equals the level of control by the most efficient enemy alone (40%). Neutral responses are most expected when both agents are equally effective and functionally redundant, sharing similar niches (Casula et al. 2006; Straub et al. 2008). Lastly, two agents have negative effects when their combined action result in lower control than that provided by the most effective agent alone (lower than 40% in the example). Negative results are expected when there are antagonistic interactions among natural enemies, such as predation, hyperparasitsm, pathogenicity and exploitative competition (Wootton 1994; Rosenheim et al. 1995). It is important to notice that antagonism between biocontrol agents does not always disrupt biological control, especially in more complex food webs and environments (Janssen et al. 2006; Okuyama 2009).

Some authors argue that functional complementarity of natural enemies can increase pest control (Casula et al. 2006; Tylianakis and Romo 2010). Accordingly, resource partitioning between biocontrol agents that clearly differ in biology (reproduction rate, morphology e.g.) and prey use patterns (foraging activity, life stage consumed e.g.) may be beneficial for pest suppression. For example, the combination of predators and pathogens may result in better control (Ramirez and Snyder 2009). Besides differences in prey/host use, predators and pathogens may also have complementary actions by acting during different times of the growing season: pathogens are often efficient only under optimal environmental conditions (Quesada-Moraga et al. 2024), and therefore, temporal releases of predators that are effective during different parts of the season could ensure control of a target pest during the other periods of the growing season (Wraight 2003).

Predatory mites and entomopathogenic fungi are examples of organisms that might give some level of niche complementarity. Predatory mites from the Phytoseiidae family are one of the most commercialized biocontrol agents of the world (Knapp et al. 2018), although in Latin America few species are commercialized (Zacarias et al. 2019). Several phytoseiid species are generalist predators (McMurtry et al. 2013), being able to survive even in the absence of a target pest (Hassell and May 1986) because they can feed on multiple sources, such as pollen, nectar and alternative prey (Settle et al. 1996; van Rijn et al. 2002; Nomikou et al. 2003). Such characteristics are desirable for biological control because it allows the establishment of these natural enemies in the crop before the target pest, and therefore, contribute to preventive control of a pest (Murdoch et al. 1985; Calvo et al. 2015; Midthassel et al. 2016). Regarding pathogens, fungi are the predominant naturally occurring pathogens in arthropod populations (Lacey et al. 2015). Entomopathogenic fungi infect a wide range of pests (Shah and Pell 2003) and can be easily mass-produced on different substrates with a variety of techniques (Jaronski and Jackson 2012). Entomopathogenic fungi are mainly used as a curative treatment to control high densities of herbivores in agroecosystems (Midthassel et al. 2016; Liu et al. 2019a), but control may occur too late to protect crops from damage (Wraight 2003). In this sense, predatory mites and pathogenic fungi might act complementarily in pest suppression (Saito and Brownbridge 2018), but the pathogen may also impact predator survival (Furtado et al. 1996; Wu et al. 2015; Liu et al. 2019b). Hence, it is important to assess the compatibility of those agents before developing a biological control program using both (Midthassel et al. 2016).

One example of a pest that could be controlled by combination of a predator and a pathogen is the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). It is a key pest of citriculture, occurs in many citrus-producing regions and can easily disperse (Bové 2006; Lewis-Rosenblum et al. 2015). It transmits *Candidatus* Liberibacter species that cause Huanglongbing (HLB), or Citrus Greening, an untreatable disease that is often difficult to detect early (Bové 2006; Gottwald et al. 2020). Citrus production has suffered major economic losses due to the occurrence of HLB. In the United States, for example, not only the production has declined, but also the number of citrus growers, juice processing industries and packing houses, and HLB has resulted in an increase in production costs (Singerman et al. 2018). It is estimated that 90% of the citrus trees in Florida were infected with HLB in 2015 (Singerman and Useche 2016). In Brazil, the disease has spread to five states and to most cities of São Paulo, the largest citrus-producing state of the country (MAPA 2024). The disease leaded to a decline in the number of citrus farms in Brazil since the first detection of HLB in 2004 (Coletta-Filho et al. 2010; Bassanezi et al. 2020). Control of the vector is a very important strategy to reduce HLB incidence and progress (Bassanezi et al. 2013). Thus, biological control can be an efficient and sustainable approach to reduce *D. citri* densities and limit the disease spread (Adami et al. 2019; Shrestha et al. 2021).

The predatory mite *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae) and the fungus *Cordyceps fumosorosea* (Wize, 1904) (Hypocreales: Cordycipitaceae) are natural enemies of *D. citri*. The generalist predator *A. herbicolus* can increase its densities on plants by feeding on pollen (Duarte et al. 2015) and, thereby, act as preventive control strategy: *A. herbicolus* released on orange jasmine plants with pollen and oviposition sites before the arrive of the pest was shown to reduce *D. citri* densities (Kalile et al. 2023). The fungal pathogen *C. fumosorosea* is currently one of the few biocontrol agents commercially available to control this pest (Mascarin et al. 2019) and is highly virulent to adults (Avery et al. 2009). However, the dissemination and efficacy of the pathogen relies on environmental conditions and host densities (Conceschi et al. 2016; Saldarriaga Ausique et al. 2017). Thus, the combination of *A. herbicolus* and *C. fumosorosea* could result in increased control of *D. citri*. Yet, the pathogen may represent a risk for the predator. Therefore, I studied the compatibility of *A. herbicolus* and *C. fumosorosea*, aiming at the control of *D. citri*. In chapter 1, I investigated the pathogenicity of *C. fumosorosea* to *A. herbicolus* juveniles and adults. In chapter 2, I studied the population dynamics of Asian citrus psyllid with both natural enemies.

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Chapter 1:

A predatory mite of the Asian citrus psyllid shows low susceptibility for an entomopathogenic fungus of the psyllid

ABSTRACT

Worldwide, citrus cultures are threatened by Huanglongbing, a fatal and untreatable bacterial disease vectored by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae). Recent studies suggest that there is potential for preventing spread of the disease through biological control of the psyllid. Entomopathogenic fungi and predatory mites are efficient biological control agents of *D. citri*. Combining predators and pathogens could enhance suppression of psyllid populations, but there is the risk of infection of predators by the fungus. Here, we investigated the pathogenicity of the fungus *Cordyceps fumosorosea* (Wize, 1904) (Hypocreales: Cordycipitaceae) for the predatory mite *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae), aiming to assess the compatibility of both natural enemies for control of the Asian citrus psyllid. We measured survival, juvenile development, oviposition and egg viability of the predators when exposed to the *C. fumosorosea* ESALQ-1296 strain. Adults and juveniles treated with the fungal spores did not show significant higher mortality than in a control treatment without fungal spores. The fungus also did not affect the developmental time from larva to adult or oviposition and egg hatch rate. This suggests that *C. fumosorosea* has no direct effects on life history traits of *A. herbicolus*, and the two species can thus be used together for biocontrol of *D. citri.* Future research could investigate if the concurrent use of *A. herbicolus* and *C. fumosorosea* enhances control of Asian citrus psyllid.

INTRODUCTION

Huanglongbing (HLB) is an untreatable and fatal disease caused by three *Candidatus* bacteria species (Garnier et al. 1984; Bové 2006). The disease is of high economic importance for citriculture due to the increase of production costs, reduction of plant productivity and the absence of commercial immune citrus varieties (Bové 2006; Munir et al. 2018; Alquézar et al. 2022). The Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is the main vector associated with HLB, is found in many citrus-producing regions and has high dispersal capacities (Bové 2006; Lewis-Rosenblum et al. 2015). Early diagnosis of the disease is important to avoid its spread (Gottwald et al. 2020), but HLB symptoms are easily confused with some other diseases and nutrient deficiencies (Shokrollah et al. 2011) and infected plants may also go through an extended asymptomatic period (Gottwald et al. 2020). Most techniques currently used to detect HLB are expensive and time consuming (Arredondo Valdés et al. 2016). Besides, the lack of taxonomic, physiological and molecular knowledge about the *Candidatus* species compromises the development of control strategies focused on the pathogen (Stewart 2012; Merfa et al. 2019). Therefore, attempts to control HLB have been largely based on control of the vector (Bassanezi et al. 2013; Li et al. 2020; Alquézar et al. 2022).

Chemical control of *D. citri* is currently most used (Belasque Jr et al. 2010; Grafton-Cardwell et al. 2013; Chen et al. 2020). However, pesticides are inefficient in preventing the introduction and dissemination of HLB to new citrus orchards (Bergamin-Filho et al. 2008; Bassanezi et al. 2013). Besides their low efficiency, pesticides cause severe hazard to pollinators (Brittain et al. 2010; Blacquière et al. 2012; Chen et al. 2017), affect populations of natural enemies resulting in pest resurgence (Janssen and van Rijn 2021), and might result in outbreaks of secondary pests (Zanardi et al. 2018). The continuous use of insecticides has also led to loss of susceptibility of *D. citri* (Tiwari et al. 2011; Chen et al. 2018, 2020, 2022).

Biological control is a promising alternative for controlling *D. citri* (Kalile et al. 2021, 2023; Shrestha et al. 2021). Several organisms are reported as natural enemies of the psyllid, including parasitoids (Chen and Stansly 2014; Bistline-East et al. 2015), predators (Michaud 2004; Qureshi and Stansly 2009; Kistner-Thomas et al. 2016; Kalile et al. 2021) and pathogens (Subandiyah et al. 2000; Avery et al. 2009; Stauderman et al. 2012; Hall et al. 2012). Fungal pathogens are currently the only biological control agent commercially available for this pest (Mascarin et al. 2019; Wendel et al. 2022). A commercial strain of *Cordyceps fumosorosea* (Wize, 1904) (Hypocreales: Cordycipitaceae) (basionym: *Isaria fumosorosea*) is highly virulent (Subandiyah et al. 2000; Avery et al. 2009; Conceschi et al.

2016) and effectively controls *D. citri* adults under laboratory conditions (Stauderman et al. 2012). In commercial groves, Asian citrus psyllid infection and mortality by *C. fumosorosea* vary along the year, depending on relative humidity, rainfall and temperature (Saldarriaga Ausique et al. 2017). Little is known about the effect of this pathogen on non-target organisms (Pell and Vandenberg 2002; Zimmermann 2008) such as other natural enemies of *D. citri*.

The parasitoid wasp *Tamaraxia radiata* (Waterson) (Hymenoptera: Eulophidae) was introduced in several countries for biological control of *D. citri* (Étienne et al. 2001; Qureshi et al. 2009). However, its effectiveness is limited due to climate factors (McFarland and Hoy 2001; Gómez-Torres et al. 2014). Besides, mass-rearing of *T. radiata* is laborious (Chen and Stansly 2014). Predators are the most diverse natural enemies of *D. citri* in various citrus producing regions, yet few studies evaluated the role of predators in suppressing *D. citri* (Hall et al. 2013; Kondo et al. 2015). Recent studies (Kalile et al. 2021, 2023) showed that the predatory mite *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae) can develop and reproduce by feeding on eggs, first and second instar nymphs of *D. citri*. It can also suppress Asian citrus psyllid densities on orange jasmine plants *Murraya paniculata* (L.) Jack (Rutaceae) when pollen and oviposition sites are added (Kalile et al. 2023).

Natural enemy assemblages can enhance suppression of herbivore densities on plants (Cardinale et al. 2003; Griffin et al. 2013; Paredes et al. 2015; Roubinet et al. 2015; Jonsson et al. 2017). According to this scenario, generalist predatory mites and entomopathogenic fungi together may control pests better than each agent separately (Midthassel et al. 2016; Liu et al. 2019a; Castillo-Ramírez et al. 2020). Predatory mites could act as a preventive control strategy, because they can be established in the crop by supplying them with alternative food before arrival of the pest (Jacobson et al. 2001b; Nomikou et al. 2002; Tuovinen and Lindqvist 2010; Calvo et al. 2015). In contrast, entomopathogenic fungi can only be successfully applied when the pest already occurs (Roberts and Hajek 1992; Jacobson et al. 2001a; Midthassel et al. 2016; Liu et al. 2019a). Therefore, integration of entomopathogens with generalist predators could provide better pest control. There is also evidence that predatory mites can transport fungal conidia to their shared prey/host (Lin et al. 2019; Castillo-Ramírez et al. 2020), which is especially important when fungal suspensions fail to reach all target pest individuals (Baverstock et al. 2009). However, there may also be negative interactions between entomopathogens and other arthropod natural enemies, and it is therefore important to assess the compatibility of these natural enemies (Midthassel et al. 2016).

Interactions between predatory mites and entomopathogenic fungi can be complex. The pathogens may be harmless to predatory mites (Wu et al. 2016b) or might affect survival, behavior and life history traits of juvenile and adult mites (Duso et al. 2008; Seiedy et al. 2012a; Wu et al. 2015; Ullah and Lim 2017). Pathogens may also have indirect effects on predator densities by reducing prey densities on plants (Roy and Pell 2000) and affecting the quality of prey (Seiedy et al. 2012b). As a first step in investigating the compatibility of a commercial strain of *C. fumosorosea* on *A. herbicolus*, we assessed the effect of the fungus on survival, development and reproduction of these predatory mites.

MATERIALS AND METHODS

Pollen

Pollen was collected from *Typha* spp. plants in Viçosa, Minas Gerais, Brazil, in 2019. It was dried in an oven for 12 hours at 40 °C and then stored in freezer at -6 °C. Small amounts of pollen were periodically removed from the freezer, put in 1.5 ml microtubes (Eppendorf) for daily use. It was dried at 60 °C for 48 h and then stored in the refrigerator (8 °C) (Kalile et al. 2021).

Asian citrus psyllid rearing

Asian citrus psyllids came from stock colonies maintained in our lab, originally collected in 2018 from orange jasmine plants on the campus of the Federal University of Viçosa (Kalile et al. 2021). Psyllids were reared on orange jasmine plants maintained in BugDorm-4f cages (50 x 50 x 100 cm). These plants, 4 to 6 months old, were obtained from Viveiro Antuérpia in Viçosa, MG. Plants were watered twice a week and adult psyllids were regularly transferred to new plants. The cages were kept in the laboratory under natural light conditions and ambient temperature.

Predator rearing

All predatory mites came from stock colonies maintained in our lab. They were collected from tomato plants in Prados (latitude: 21.0300000 S; longitude: 44.0404700 W), Minas Gerais, Brazil (Cardoso et al. 2024). They were reared on arenas consisting of a black plastic sheet (15 x 10 cm) placed on top of a wet sponge (h = 4 cm) centered in a plastic tray (29 x 14 x 4 cm) containing water. The edges of the plastic sheet were covered with moistened cotton wool to prevent mites from escaping, and also serving as a source of water (McMurtry and Scriven 1965). Pieces of tent-shaped plastic sheet (1.5 x 1.5 cm) were randomly placed on the arenas to serve as shelters. Small pieces of cotton wool were placed below each tent to serve as sites of oviposition (Marcossi et al. 2020). Predators were fed with cattail *Typha* spp. pollen two times a week. Arenas were kept in climate-controlled rooms (25 ± 1 °C, 70 ± 10% RH, 12:12 L:D). Cotton wool with eggs was placed on new arenas to obtain mites with approximately the same age for the experiments.

Fungal inoculum

We used the *C. fumosorosea* ESALQ-1296 strain, provided by Koppert Brasil (commercial formulation Challenger®). We prepared a conidial suspension in a solution of distilled water with polyoxyethylene sorbitan monooleate (Tween 80®, Vetec, Duque de Caxias, RJ, Brazil) at 0.01% (v/v) as surfactant. The conidial suspension concentration was standardized at 1 x 10^7 viable conidia/mL for all experiments, according to recommendations for the control of Asian citrus psyllid in Brazil. To obtain this concentration, 60 µL of the commercial formulation was mixed with 15 mL of the distilled water + surfactant solution.

Pathogenicity for Asian citrus psyllid adults

The efficacy of *C. fumosorosea* ESALQ-1296 strain has been proven before. This test was designed to validate our experimental method, which was adapted from Avery et al. (2009). Petri dishes (Ø = 9 cm and h = 1 cm) were lined with moistened filter paper. We cut orange jasmine leaflets in discs (Ø = 1,71 cm) and placed 5 discs in a single dish. We placed discs with their abaxial side up and equally spaced in cross-like arrangement. A conidia suspension was prepared as above (**Fungal inoculum**) and 3 mL of the resulting suspension was transferred to a Potter spray tower using a 100-1000 µL micropipette. We sprayed this volume on each dish (a total of 24 dishes) with the spray tower calibrated at an atomization pressure of 10 lb/in^2. A control group of dishes (n = 15) was sprayed with only distilled water with surfactant. Sprayed discs were allowed to air dry for 3 hours. The fungus was sprayed on leaf discs and not directly on adult psyllids because these would escape during spraying. To collect *D. citri* adults (aged 7-14 days, mixed sex) from rearing cages, we used a clear PVC hose (Ø = 1 cm) connected to a pipette tip (1000 µL) with the tip cut to enlarge the opening to allow psyllids to pass. A mesh (90 µm) was placed between the hose and the pipette tip and psyllids were sucked into the tip. Subsequently, the tip was removed from the hose and sealed at both ends with Parafilm M® (Bemis Flexible Packaging, Neenah, WI, USA). The pipette tip was then placed in a refrigerator at 5 ° C for 5 minutes to immobilize the psyllids.

Subsequently, the Parafilm was removed and single psyllids were transferred to each Petri dish using a fine brush. Immediately after releasing the psyllids, dishes were sealed with plastic cling film and moved to a climate-controlled room (25 ± 1 °C, 70 ± 10% RH, 12:12 L:D). Mortality was assessed daily for 7 days. We also checked if dead psyllids showed signs of fungal infection. If mycosis was observed, the infected psyllid was transferred with tweezers to a Petri dish (Ø = 5 cm and h = 1 cm) containing PDA media to allow fungal growth. After seven days, slides of the fungal structures were prepared in fuchsin acid (0.03%). Fungi were identified at the Insect-Microorganism Interactions Laboratory at the Federal University of Viçosa based on morphological characteristics of conidia and conidiophores. The survival of psyllid females exposed to *C. fumosorosea* was compared to the control with a Kaplan-Meier analysis of the 'survival' package of R (Therneau 2022). All statistical analyses were performed using software R version 4.2.2 (R Core Team 2022).

Pathogen effects on survival and oviposition of adult predators

In order to spray mites with conidia, we placed a black plastic sheet (Ø = 7 cm) on top of a wet sponge (Ø = 9 cm and h = 1 cm) inside a Petri dish of the same size as above. The edges of the arena were surrounded with wet cotton wool to prevent mites from escaping. Up to 18 gravid females of *A. herbicolus*, aged 10 to 12 days after egg hatching, were placed on the plastic sheet. A conidia suspension was prepared as above (**Fungal inoculum**). We sprayed 1 mL of the conidia suspension on each Petri dish with the same atomization pressure as above (**Pathogenicity for Asian citrus psyllid adults**). A control treatment consisted of spraying only distilled water with surfactant. We sprayed a total of 33 females in the treated group and 35 females in the control group. All groups were sprayed within the same hour. After spraying, females were isolated individually in black plastic arenas (Ø = 5 cm, h = 1.4 cm) containing a piece of wet cotton wool as source of moisture, ample cattail pollen as food and arenas were covered with a transparent lid. Once per day, we assessed survival and the number of eggs laid by each female for a period of 10 days, and females were moved to a new arena using a fine brush. The survival of females exposed to *C. fumosorosea* was compared to the control with a Cox proportional hazards model of the 'survival' package. The average oviposition rate per female per day was analyzed with a generalized linear model (GLM) using Gausian error distribution with treatment as factor. For completeness, we present oviposition through time.

Pathogen effects on egg viability

We placed up to 10 predator eggs (< 24 h old) on black plastic arenas as above. The arenas were sprayed with a conidia suspension or with water with surfactant as above (**Pathogen effects on survival and oviposition of adult predators**). A total of 40 eggs (4 arenas) was sprayed per treatment. After spraying, we placed a piece of wet cotton wool inside the arenas, which were covered with a transparent lid. Eggs were not transferred to new arenas to prevent damaging eggs due to handling. We assessed the number of hatched eggs once per day for three days. Eggs usually hatch in less than two days (Reis et al. 2007). Eggs that did not hatch were considered unviable. Mites that died during hatching were also considered unviable. The proportion of hatched eggs through time was analyzed with a generalized linear mixed-model with a binomial error distribution (GLMM) with the proportion of hatched eggs and time as factors, replicate as random variable and a correction for overdispersion. GLMM was performed using 'glmer' function from the package 'lme4' (Bates et al. 2023).

Pathogen effects on juvenile development and survival

To evaluate the effects of *C. fumosorosea* on the survival and development of *A. herbicolus* juveniles, we sprayed two groups of 16 and 15 mites with a conidia suspension as above (**Pathogen effects on survival and oviposition of adult predators**), and two control groups of 15 and 21 nymphs were sprayed with water and surfactant. Because larvae are vulnerable to manipulation, we used protonymphs (3 days since hatching). As above, we transferred nymphs to black plastic arenas with a piece of moist cotton wool and ample pollen as food. Juveniles were daily transferred to new arenas with new pollen. Survival and development stages were assessed daily until mites reached adulthood and started laying eggs. The effect of fungal pathogen on the survival and juvenile development of mites was analyzed with a Cox mixed effects model with spraying as fixed effect.

RESULTS

Pathogenicity for Asian citrus psyllid adults

The survival of *D. citri* was significantly reduced by exposure to leaflets treated with *C. fumosorosea* (Fig. 1A; Kaplan-Meier: $\text{Chi}^2 = 28.9$; d.f. = 1; $p < 0.0001$). None of dead psyllids of the control group showed mycosis, whereas all dead treated psyllids showed growth of fungal structures four days after spraying (Fig. 1B). The fungus was confirmed to belong to the *Cordyceps* genus.

Survival and oviposition of adult predators

The survival of *A. herbicolus* adults was not affected by the exposure to *C. fumosorosea* (Fig. 2A, Cox proportional hazards: χ^2 = 1.28; d.f. = 1; p = 0.295). The fungal pathogen had no negative effect on the oviposition of the predators (Fig. 2B, GLM: $F_{1,62}$ = 0.611; p = 0.437). Growth of fungal structure was observed on two dead treated mites (Fig. 3).

Egg viability

Viability of *A. herbicolus* eggs was not significantly affected by the exposure to the pathogen (Fig. 4, GLMM: Chi^2 = 0.450; d.f. = 1; p = 0.481). No significant effect was observed of the interaction between treatment and time (Chi^2 = 2.23; d.f. = 1; p = 0.135), indicating that exposure to *C. fumosorosea* had no impact on hatching time.

Juvenile development and survival

Exposure to *C. fumosorosea* conidia did not significantly affect the juvenile development of the predator (Fig. 5A, Cox mixed effects: Chi^2 = 1.63; d.f. = 1; p = 0.201). Nymphs of *A. herbicolus* survived equally high in both groups (Fig. 5A, Cox mixed effects: Chi^2 = 0.037; d.f. = 1; p = 0.847). Growth of fungal structure was observed in one treated nymph (Fig. 5B).

DISCUSSION

A strain of *C. fumosorosea* that is highly virulent against the Asian citrus psyllid did not affect survival, reproduction or development of *A. herbicolus*, one of the natural enemies of *D. citri*. To the best of our knowledge, the present research is the first to investigate the compatibility of *A. herbicolus* with a pathogen. *C. fumosorosea* had been tested before with other phytoseiid predator species, leading to some mortality of mites (Castillo-Ramírez et al., 2016).

In the experiment with *D. citri*, adults from control group showed a strong decrease in survival after six days (Fig.1). This was not because of infection with *C. fumosorosea* because mycosis did not occur on dead adults from this control group. Perhaps adults did not feed on leaf discs and starved. Indications for this are that we found no honeydew or other excretions, whereas adults of *D. citri* produce these while feeding (Ammar et al. 2013). *D. citri* can survive up to five days without food and water, depending on temperature (Zhang et al. 2019), so the increased mortality after 6 days may well have been caused by starvation. Nevertheless, this shows that the experimental set-up used here can be optimized, for example by transferring the adults to fresh plant material sometime after exposure.

The experimental methods for the psyllids and predators were not the same because of differences in their morphology and biology. There were three clear differences between methods: (1) *A. herbicolus* was directly sprayed with fungus, while *D. citri* was exposed to treated leaves; (2) psyllids had longer exposure time to conidia than predators and (3) the volume of spraying used was different. Thus, we cannot be sure whether results from **(Pathogenicity for Asian citrus psyllid adults)** validate the results from experiment with predators, but literature may support our methods. Shehzad et al. (2021) showed that direct spraying fungi conidia on an insect caused higher pathogenicity in relation to exposure to treated leaves. Regarding exposure time, Seiedy et al. (2015) showed that a fungal strain was pathogenic when directly sprayed on a phytoseiid mite, but not when the mites were exposed for several days to leaves treated with the pathogen. Considering this result, it is possible that, in our study, fungus would not be pathogenic to *A. herbicolus* even if mites were exposed to treated leaf discs for several days. Lastly, predators were exposed to a lower spray volume to reduce risks of accidental mortality during spraying. Other authors showed that treating mites (Seiedy et al. 2012a; De Oliveira et al. 2020) and insects (Ezzati-Tabrizi et al. 2009; Martins et al. 2016) with entomopathogenic fungi using the same spray volume and pressure as ours did induce infection and mortality. In short, lack of significant effects on the predators is probably not due to using a wrong methodology.

Several studies with other pathogen and phytoseiid species (Jacobson et al. 2001a; Wekesa et al. 2006; Wu et al. 2014, 2016b; Zhang et al. 2015; Lin et al. 2017; Sun et al. 2018) also showed high survival of other species of phytoseiid mites. The high survival of juveniles found here is perhaps not surprising because ecdysis causes fungi conidia to be shed and this can prevent infection of immatures of some arthropods (Vey and Fargues 1977; Wekesa et al. 2006). Moreover, *A. herbicolus* has a short developmental time with several stages (protonymph, deutonymph, adult) so ecdysis occurs frequently in this species. Our results are also consistent with studies that showed no effect of entomopathogenic fungi on the hatch rate of eggs from other predatory mite species (Duso et al. 2008; Midthassel et al. 2016; Ullah and Lim 2017; Liu et al. 2019b). In contrast, other studies did report detrimental effects of entomopathogenic fungi on phytoseiid mites (Furtado et al. 1996; Seiedy et al. 2015; Dogan et al. 2017). Perhaps *A. herbicolus* was not infected by *C. fumosorosea* because of host specificity of the fungus strain. In our study, three treated dead mites (two adults and one nymph) showed mycosis (Fig. 3 and Fig. 5B), but we do not know whether this was *C. fumosorosea*. Even if it was, the high predator survival and the low number of cadavers colonized by the fungus suggest that *A. herbicolus* may largely be invulnerable for the fungus strain used here. It remains to be investigated whether this also holds for other strains of *C. fumosorosea* because different strains of the same fungus species may cause distinct pathogenic responses in phytoseiid mites (Ferron 1978; Goettel et al. 1990; Brodeur 2012; Seiedy et al. 2015).

Pathogens can also affect non-target organisms in other ways than through causing mortality. For example, negative effects of fungi on developmental time and oviposition of predatory mites were reported before (Dogan et al. 2017; Ullah and Lim 2017; Liu et al. 2019b), but such effects were not observed here. However, the two potential biocontrol agents can also have indirect negative effects on each other, for example through exploitative competition for *D. citri*. Under optimal conditions, *C. fumosorosea* can drastically reduce psyllid densities and this could be harmful for the predator. However, *A. herbicolus* can survive and reproduce on alternative food sources (Duarte et al. 2015; Marcossi et al. 2020), hence, the supply of alternative food could enable the persistence of predators on plants treated with the fungus.

Furthermore, the pathogen could reduce the nutritional quality of the prey and thus affect predator biology (Flick et al. 2016). Wu et al. (2014) showed that a fungus had no detrimental effect when directly applied to *Neoseiulus barkeri* (Hughes, 1948) (Acari: Phytoseiidae), but life history traits of predators were affected when they fed on prey infected with the fungus (Wu et al. 2015). *Neoseiulus barkeri* also preyed more when infected prey was offered, possibly as a consequence of lower prey quality. Therefore, it is important to investigate whether feeding on infected eggs and nymphs of *D. citri* alters the survival, development, reproduction and predation of *A. herbicolus*. Additionally, it could be investigated whether *A. herbicolus* has a preference for infected or uninfected eggs and nymphs, which would strengthen the combined control of *D. citri* with both control agents. Indeed, some predatory mite species were reported to avoid cues from prey infected with fungus (Seiedy et al. 2013) or leaves with spores (Wu et al. 2018). Predatory mites that do not avoid leaves with spores may act as vectors of entomopathogenic fungi by delivering conidia to the shared pest (Lin et al. 2019). Some studies showed that using mites to vector fungal conidia enhanced mortality of the pest (Zhang et al. 2015; Castillo-Ramírez et al. 2020). However, these authors did not study the effects of this on control of the pest. Further research is required to verify the capability of *A. herbicolus* in vectoring *C. fumosorosea* conidia and how this relation could impact dynamics of *D. citri* on plants.

The actions of assemblages of natural enemies can lead to various results in pest control (Ferguson and Stiling 1996; Wraight 2003), from increased to decreased pest densities. The results presented here make it less likely that combining *A. herbicolus* and *C. fumosorosea* would negatively affect biological control of *D. citri*. The question then remains whether the use of both control agents will increase control of *D. citri* relative to control provided by each agent separately. Although some authors related that the combined use of predatory mites and entomopathogenic fungi resulted in better pest control (Saito and Brownbridge 2018; Hernández-Valencia et al. 2024), there are also examples where control did not improve (Wu et al. 2016a). Therefore, further experiments testing *A. herbicolus* and *C. fumosorosea* isolated and in combination are required to answer if their combined use would result in a better control of *D. citri*.

We showed that an entomopathogenic fungus strain that is efficient against *D. citri* had no direct detrimental effect on a predatory mite that also attacks this psyllid. Although this is a prerequisite for using the predator and the fungus together for biocontrol of *D. citri*, further research is required to see their single and combined effects on pest control.

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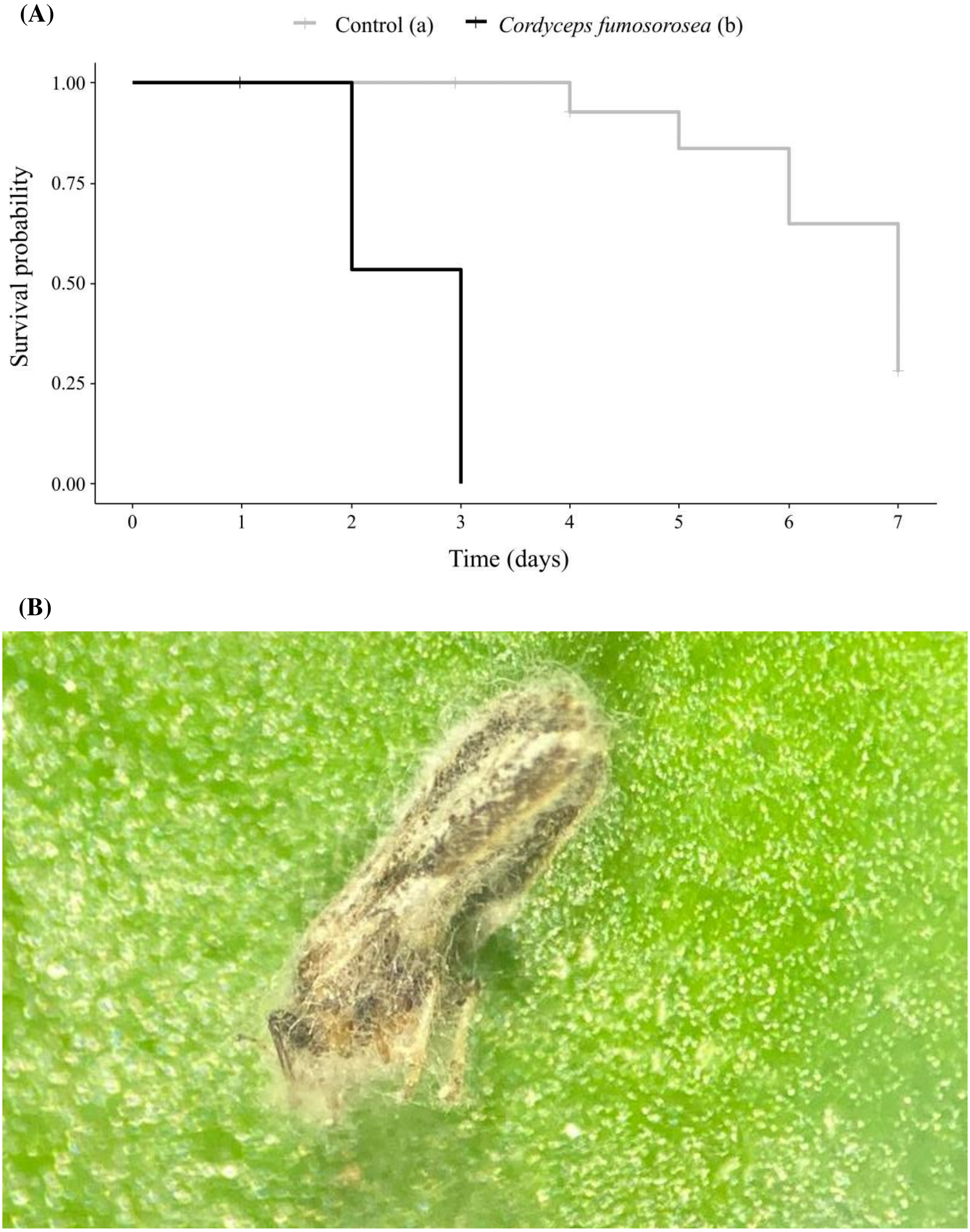

Figure 1: **(A)** Cumulative survival probability of *Diaphorina citri* adults either exposed to leaf discs treated with *Cordyceps fumosorosea* conidia or not (Control). Exposure to conidia had a significant effect on the survival of adults (Kaplan-Meier). **(B)** Fungal growth on a dead adult *Diaphorina citri* after exposure to leaf discs treated with *Cordyceps fumosorosea*.

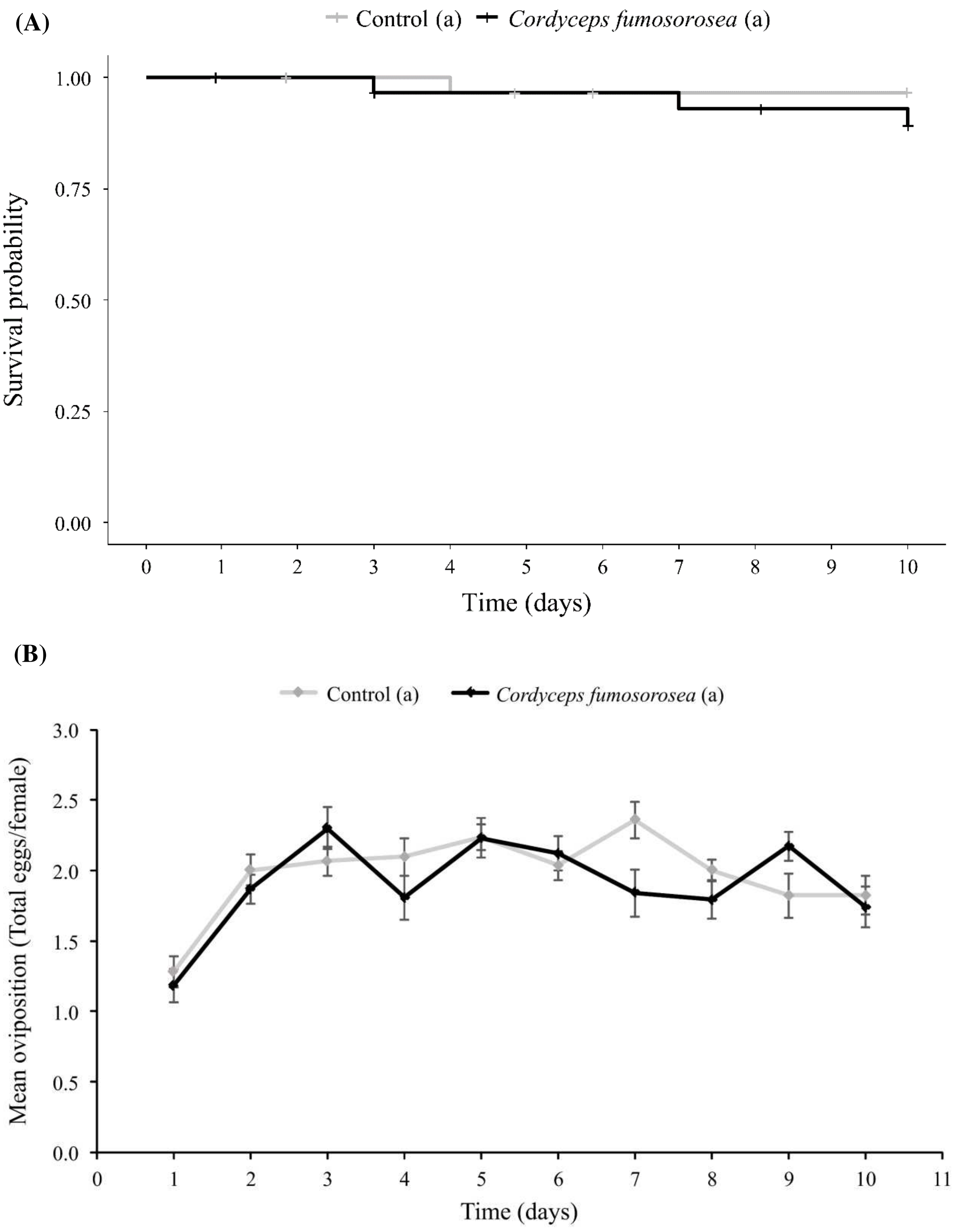

Figure 2: **(A)** Cumulative survival probability of *Amblyseius herbicolus* adults that were either sprayed with *Cordyceps fumosorosea* conidia or not (Control). Exposure to conidia had no significant effect on the survival of adults (Cox proportional hazards). **(B)** Mean oviposition rate (± SE) of *Amblyseius herbicolus* either exposed to *Cordyceps fumosorosea* conidia or not. Exposure to the pathogen had no significant effect on predator oviposition (GLM).

Figure 3: Fungal growth on body of one dead *Amblyseius herbicolus* adult exposed to *Cordyceps fumosorosea*.

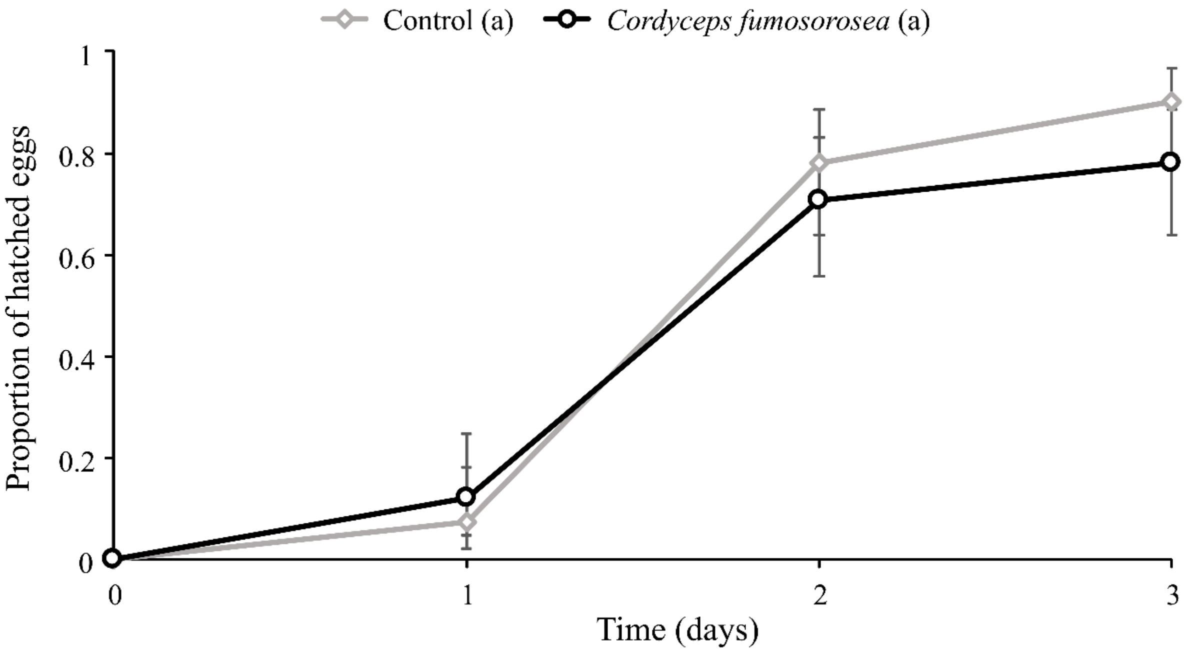

Figure 4: Viability of *Amblyseius herbicolus* eggs either exposed to *Cordyceps fumosorosea* conidia or not. Shown is the cumulative proportion hatched eggs as a function of time. Exposure to conidia had no significant effect on proportion of hatched eggs. Error bars are binomial confidence intervals (95%).

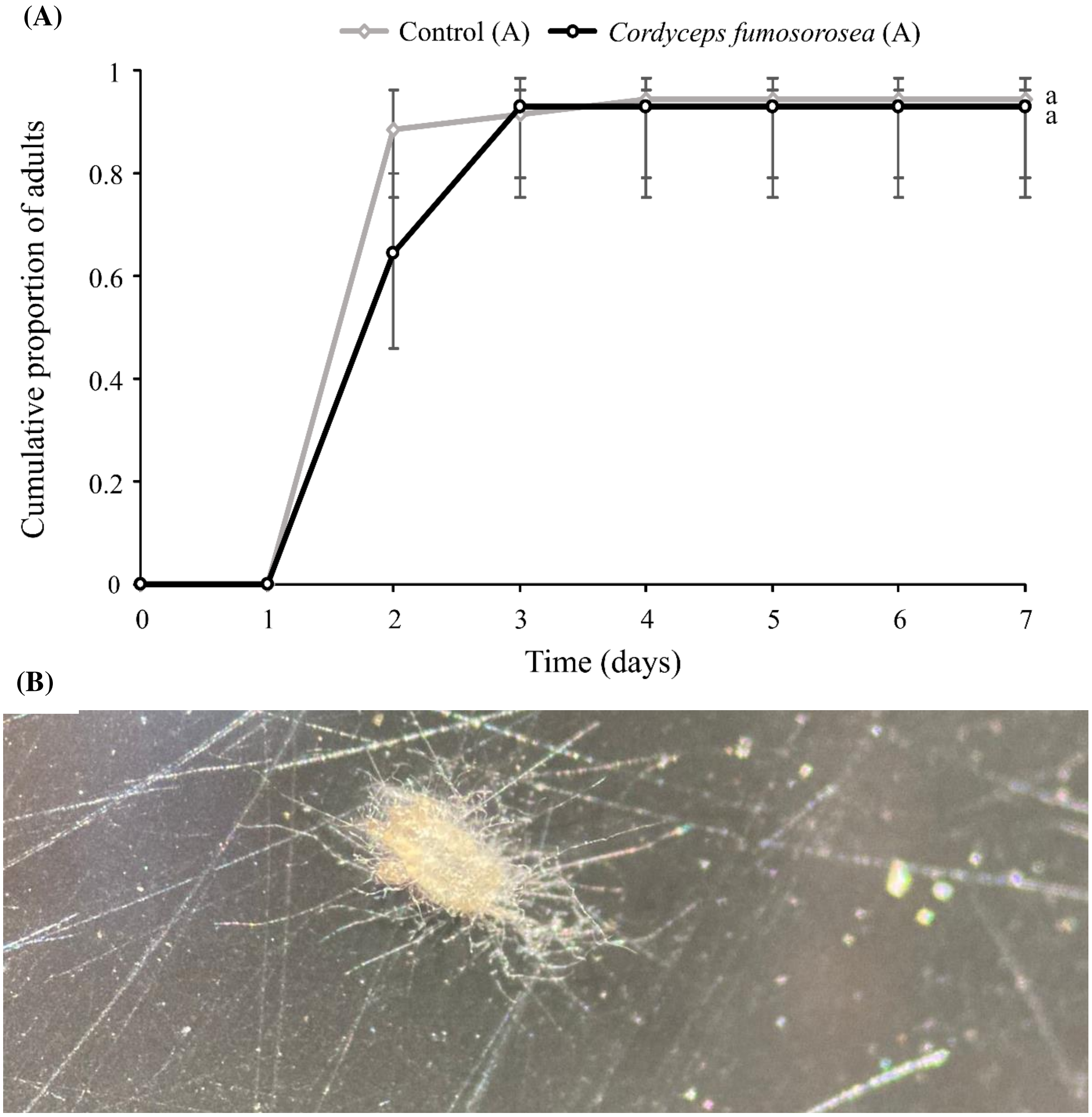

Figure 5: **(A)** Development and survival of *Amblyseius herbicolus* juveniles either sprayed with *Cordyceps fumosorosea* conidia or not (Control). Shown is the cumulative proportion of mites that reached adulthood as a function of time. Survival is the final proportion of adults (at 7 days). Treatment with conidia had no significant effect on the development (uppercase letters) and survival (lowercase letters). Error bars are binomial confidence intervals (95%). **(B)** Fungal growth on body of dead *Amblyseius herbicolus* nymph exposed to *Cordyceps fumosorosea*.

Chapter 2:

Concurrent use of a predator and a pathogen for biological control of the Asian citrus psyllid

ABSTRACT

The Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) is a pest of great importance in citrus, because it acts as a vector of Huanglongbing, the most destructive citrus disease. It is attacked by a great diversity of natural enemies, among others, the predatory mite *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae) and the fungal pathogen *Cordyceps fumosorosea* (Wize, 1904) (Hypocreales: Cordycipitaceae). Recent studies have suggested that combing predators and pathogens may enhance biological control of a pest through functional complementarity. Here, we studied the population dynamics of *D. citri* on plants with both control agents separately and combined. Plants were supplemented with pollen and cotton threads for the maintenance of predators. Predator were released a week before the release of *D. citri*, while the pathogen was sprayed three times after this release. The lowest densities of *D. citri* were observed on plants treated only with the entomopathogenic fungus and plants treated with both predators and pathogen. However, predators were not found back on any plant at the end of the experiment, and therefore, densities of *D. citri* on plants treated only with *A. herbicolus* did not differ from those on the control plants. We discuss limitations of the methods that may have caused the extinction of the predators.

INTRODUCTION

Biological control of single pest species is often affected by interactions among multiple natural enemy species (Messelink et al. 2011). The effect of those interactions on pest control has been studied by many authors and different results were achieved (Denoth et al. 2002; Cardinale et al. 2003; Letourneau et al. 2009). The presence of multiple enemy species can contribute to enhance suppression of herbivore densities (Jonsson et al. 2017), but biological control may also be disrupted due to negative interactions among the enemies such as predation, interference and competition (Rosenheim et al. 1995; Messelink et al. 2011). Enemy assemblages can also have non-additive effects on the suppression of herbivore densities, resulting in a level of control equal to that provided by the most effective agent alone (Ferguson and Stiling 1996). These effects can occur due to the cancelling out of positive and negative effects, weak interactions between agents, high efficiency of a single agent or functional redundancy (Casula et al. 2006; Straub et al. 2008; Letourneau et al. 2009). Thus, some authors argue that success from combining enemies in pest control can be achieved through functional complementarity, when enemies have different patterns of pest consumption (Casula et al. 2006). Predators and pathogens, for example, are promising complementary agents (Ramirez and Snyder 2009), although their concurrent use may to lead to different results on pest suppression (Wu et al. 2016; Saito and Brownbridge 2018; Vázquez-Benito et al. 2022). In short, the success of combining biocontrol agents is context-dependent (Tylianakis and Romo 2010), because complexity of the food web, behavior of enemies and pests, habitat composition and temporal scale also play important roles in biological control (Janssen et al. 1998, 2006, 2007; Okuyama 2009). Therefore, investigating multi-enemy systems can provide further information on the relation between community structure and pest population dynamics (Hassell and May 1986). Here, we investigated the effects of a predator and a pathogen on the population dynamics of the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae).

This psyllid is a key pest of citrus and transmits *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter americanus, which are bacteria species associated with Huanglongbing (HLB), the most destructive citrus disease (Bové 2006). *Candidatus* Liberibacter asiaticus is the most virulent pathogen associated with *D. citri* (Wang et al. 2017; Alquézar et al. 2022), and both pathogen and vector are widespread in several important citrus-producing regions (Lewis-Rosenblum et al. 2015; Munir et al. 2018; Merfa et al. 2019). There are no commercial citrus varieties that are resistant to HLB (Munir et al. 2018), and several varieties are considered important hosts of the vector (Meng et al. 2022). Control of the vector has therefore mainly focused on the attempt to avoid HLB spread (Grafton-Cardwell et al. 2013). Citrus plants infected with HLB may go through an asymptomatic period that lasts for months or even years, delaying detection (Gottwald et al. 2020). Symptoms are similar to zinc deficiency and other diseases (Gottwald et al. 2007; Shokrollah et al. 2011), and diagnosis of the disease is therefore difficult. Both symptomatic and asymptomatic infested plants may act as a source of inoculum for pathogen transmission. Plants become infectious less than 15 days after being infected by *D. citri* (Lee et al. 2015). *D. citri* can survive on host plants other than commercial varieties of citrus. The ornamental plant orange jasmine *Murraya paniculata* (L.) Jack (Rutaceae) is an important host for *D. citri* (Meng et al. 2022), although it is no suitable host for HLB (Lopes et al. 2010).

Recent studies have shown promising results of biological control of the psyllid (Shrestha et al. 2021; Kalile et al. 2023). Several organisms are natural enemies of *D. citri*, contributing to the reduction psyllid densities, predominantly in organic orchards (Shrestha et al. 2021), where their performance is not impeded by pesticides. Several arthropod predators are associated with *D. citri*, such as ants, ladybeetles, mirid bugs, lacewings, hoverflies, spiders and predatory mites (Qureshi and Stansly 2009; Kistner-Thomas et al. 2016; Kalile et al. 2021). Other mortality factors of *D. citri* are parasitism by the parasitoid *Tamaraxia radiata* (Waterson) (Hymenoptera: Eulophidae) (Chen and Stansly 2014; Bistline-East et al. 2015) and infection by fungal pathogens (Subandiyah et al. 2000; Hall et al. 2012; Awan et al. 2021). Hence, understanding how different natural enemies interact is fundamental for successful biocontrol of *D. citri*.

Among the diversity of D. *citri* enemies, the predatory mite *Amblyseius herbicolus* (Chant, 1959) (Acari: Phytoseiidae) is a promising biocontrol agent (Kalile et al. 2021, 2023) and a strain of the fungus *Cordyceps fumosorosea* (Wize, 1904) (Hypocreales: Cordycipitaceae) is commercially available for control of the psyllid (Mascarin et al. 2019). The generalist predatory mite can be found on citrus and orange jasmine plants and is able to develop and reproduce by feeding on eggs, first and second instar nymphs of *D. citri* (Kalile et al. 2021, 2023). It can also develop and reproduce by feeding on alternative food sources such as pollen and nectar (Rodríguez-Cruz et al. 2013; Duarte et al. 2015; Marcossi et al. 2020; Iasczczaki et al. 2024). Thus, populations of *A. herbicolus* can be established on plants before the arrival of the target pest by supplying them with alternative food, thus possibly preventing plant infestation by pests (Duarte et al. 2015; Kalile et al. 2023). The fungus *C. fumosorosea* is a highly virulent pathogen of *D. citri* (Avery et al. 2009; Conceschi et al. 2016) and effectively controls *D. citri* adults (Stauderman et al. 2012; Saldarriaga Ausique et al. 2017), although it infects all psyllid life stages (Avery et al. 2009). However, the success of *C. fumosorosea* in infecting *D. citri* requires optimal environmental conditions (Saldarriaga Ausique et al. 2017), and dissemination of the pathogen is also influenced by densities of hosts (Wraight 2003; Conceschi et al. 2016). Hence, these fungi can be used curatively, after plants have become infested with *D. citri*, and *A. herbicolus* can be used preventively (Midthassel et al. 2016; Liu et al. 2019). Our previous results (Chapter 1) showed that *C. fumosorosea* did not cause detrimental effects on *A. herbicolus*, making it unlikely that simultaneous use of both agents would lead to negative effects on the control of *D. citri*. To study this, we compared the capacity of *A. herbicolus* and *C. fumosorosea* in suppressing Asian citrus psyllid densities when agents were applied alone or together by quantifying the population dynamics of *D. citri* on orange jasmine plants.

MATERIALS AND METHODS

Plant material

Orange jasmine plants were used in the experiments and for *D. citri* rearing. Pesticide-free plants, 4 to 6 months old, were obtained from Viveiro Antuérpia near Viçosa, Minas Gerais. Plants were regularly fertilized with NPK 18-04-18 and pruned to stimulate the production of shoots, which are used as oviposition sites by *D. citri*.

Asian citrus psyllid rearing

Asian citrus psyllids came from stock colonies maintained at the Laboratory of Acarology, collected from orange jasmine plants on the campus of Federal University of Viçosa. They were reared on orange jasmine plants maintained in BugDorm-4f cages (50 x 50 x 100 cm). Plants were watered twice a week, and adults were regularly transferred to new plants. The cages were kept in the laboratory under natural light conditions and ambient temperature.

Predator rearing

We used mites that were collected from tomato plants in Prados (latitude: 21.0300000 S; longitude: 44.0404700 W), Minas Gerais, Brazil (Cardoso et al. 2024). Mites were reared according to Cardoso et al. (2024). In short, arenas consisting of a black plastic sheet (15 x 10 cm) were placed on the top of a wet sponge (h = 4 cm) centered in a plastic tray (29 x 14 x 4 cm) containing water. The edges of plastic sheets were surrounded by moistened cotton wool to prevent mites from escaping, and also serving as a source of water (McMurtry and Scriven 1965; Cardoso et al. 2024). Pieces of tent-shaped plastic sheet (1.5 x 1.5 cm) were randomly placed in the arenas to serve as shelters. Small pieces of cotton wool were placed below each tent to serve as oviposition sites. Cattail *Typha* spp. pollen was provided to the mites on the arenas two times a week. Arenas were kept in climate-controlled rooms (25 ± 1 °C, 70 ± 10% RH, 12:12 L:D). Cotton wool with eggs were placed on new arenas to obtain mites with approximately the same age for the experiments (Marcossi et al. 2020).

Pollen

Pollen used for predator rearing and experiments was collected from *Typha* spp. plants in Viçosa, Minas Gerais, Brazil, in 2019. It was dried in an oven for 12 hours at 40 °C and then stored in freezer at -6 °C. Small amounts of pollen were periodically removed from the freezer, put in 1.5 ml microtubes (Eppendorf) for daily use. It was dried at 60 °C for 48 h and then stored in the refrigerator (8 °C) (Kalile et al., 2021).

Fungal inoculum

We used the *C. fumosorosea* ESALQ-1296 strain provided by Koppert Brasil (commercial formulation Challenger®). We prepared a conidial suspension with a concentration of 1 x 10^7 viable conidia/mL, according to recommendations for the control of the Asian citrus psyllid in Brazil. To obtain this concentration, we prepared 500 mL of a solution containing water and the surfactant polyoxyethylene sorbitan monooleate (Tween 80®, Vetec, Duque de Caxias, RJ, Brazil) at 0.01% (v/v) and poured the solution in the tank of a knapsack sprayer SS G2 (Brudden, Pompeia, SP, Brazil). Then, we added 2 mL of the formulation to the tank.

Population dynamics experiment

We evaluated the effects of the interaction between the predatory mite and the pathogen on Asian citrus psyllid densities in a population dynamics experiment adapted from Kalile et al. (2023). The dynamics of *D. citri* was followed on *M. paniculata* plants receiving one of four treatments: (1) plants with *A. herbicolus*; (2) plants sprayed with *C. fumosorosea* conidia; (3) plants with predators and sprayed with conidia; (4) plants without predators and pathogen. We replicated each treatment on five plants. The experiment was conducted in a greenhouse.

Orange jasmine plants (about 20-25 cm high) with growing tips were transplanted into plastic pots (Fig 1A; h = 17 cm and Ø ≈ 20 cm) and were fertilized with NPK 18-04-18. The soil was covered with a circular black plastic sheet with an opening for the stem. The objective of covering the soil was to help on the observation of predators and dead psyllids (Kalile et al. 2023). Pots with plants were put inside cages consisting of two tubular structures made of transparent acetate sheets (0.25 mm thick). The first tubular structure (Fig. 1B; h = 25 cm, C = 60 cm and Ø ≈ 20 cm) surrounded the pot and was fixed in a black plant saucer (Ø = 20 cm), sealing the bottom part of the cage. The second tubular structure (Fig. 1C; h = 50 cm, C = 60 cm and Ø ≈ 20 cm) covered the aerial plant part and could be removed during assessments. The center of the second tubular structure had an opening (10 x 10 cm) covered with mesh (90 µm) to promote air circulation. The top of the second tubular structure was also sealed with a mesh (90 µm), while its bottom was placed between the pot and the first tubular structure. The cages were placed inside a plastic tray (47 × 23 × 9.5 cm) containing water and neutral detergent to prevent invasion of other organisms in the cage.

In the treatments with predatory mites, we placed six gravid *A. herbicolus* females (age 10-12 days) on the plants using a fine brush. We provided cattail pollen and two cotton threads (5 cm long) on plants as alternative food and oviposition sites, respectively (Kalile et al. 2023). Plants of treatments without predator also received pollen and cotton threads. All plants were irrigated twice a week. Due to the low amount of pollen available, plants of treatments without mites received pollen once a week, while plants with predator received pollen twice a week. We sprinkled pollen (about 0.6 mg) on plants using a fine a brush. Cotton threads were substituted nearly a month after the release of mites.

We released fifteen *D. citri* adults on each plant five days after the release of predators. To collect adults from the rearing cages, we used a clear PVC hose (Ø = 1 cm) connected to a pipette tip (1000 µL) with an enlarged opening at the tip to allow psyllids to pass. A mesh (90 µm) was placed between the hose and the pipette tip. After sucking up adult psyllids, the pipette tip was removed from the hose and sealed at both sides with Parafilm M® (Bemis Flexible Packaging, Neenah, WI, USA). The sex of the adults was assessed under a Stemi 508 binocular stereomicroscope (25× magnification; Zeiss, Oberkochen, Germany), and consisted on average of 11 females and 4 males per tip. For the release, the top part of the cages was lifted and the pointed side with the parafilm was inserted into the soil through the opening of the plastic sheet close to the base of the stem. The parafilm was removed from the larger opening and *D. citri* adults were allowed to infest the plants.

One week after the release of the *D. citri* adults, plants of the respective treatments were sprayed with 25 mL of a suspension of *C. fumosorosea*, prepared as explained above, taking into account equipment spray pressure and application rate. A pilot test showed that this volume was enough to cover the entire plant. Plants of the other two treatments were each sprayed with 25 mL of water + surfactant solution. To avoid fungal contamination, treatments without *C. fumosorosea* were sprayed first. Spraying was repeated sixteen and thirty days after the first spraying, according to recommendations for *D. citri* control with *C. fumosorosea* (Koppert do Brasil Holding SA 2023).

We counted the number of *D. citri* adults and 4th and 5th instar nymphs once per week and checked for the presence of psyllid eggs on plant buds. We checked the presence of *A. herbicolus* through visual inspection of plants and we used a stereomicroscope to check for the presence of predator eggs on the cotton threads serving as oviposition substrate. After checking, cotton threads were discarded. Forty-four days after the release of *D. citri*, all plants were destructively sampled, counting all developmental stages of *D. citri* and *A. herbicolus*. Plants showed a large variation in size, thus we also noted plant height (ranging from to 20 to 56 cm) and number of buds (ranging from 6 to 30). We collected dead psyllids that showed mycosis, transferred them to PDA culture media to allow growth of fungi structures for seven days. Subsequently, microscope slides of the fungus were prepared and stained with fuchsin acid (0.03%). The morphology of reproductive fungus structures was observed with an Eclipse E200 microscope (Nikon Instruments Inc., Melville, NY, USA) to determine fungal genus. Fungal identification was carried out at the Insect-Microorganism Interactions Laboratory at the Federal University of Viçosa.

We compared the numbers of *D. citri* on plants (adults plus 4th and 5th instars, log(x+1) transformed) among treatments through time with a linear mixed effects model (LME) using the ‘nlme’ package (Pinheiro et al. 2023), with treatment, time and their interaction as factors. Individual plants were entered as random factor to correct for repeated measures. The significance of factors and interactions was tested with a likelihood ratio test. Contrast among treatments per each date were calculated using the ‘emmeans’ package (Lenth 2023) with the Tukey method. After the destructive counting, the total numbers all life stages of *D. citri* were analyzed with generalized linear model (GLM) with a quasi-Poisson distribution with treatment, number of buds and their interaction as factors. The significance of factors and interactions was tested with a likelihood ratio test and non-significant factors were excluded from the analysis. Contrasts among treatments were calculated using ‘emmeans’. All statistical analyses were performed with R version 4.2.2 (R Core Team 2022).

RESULTS

The interaction between treatment and time was significant for densities of psyllid during temporal samplings (Fig. 2B; LME: likelihood ratio = 15.2, df = 10, p = 0.002). Densities of psyllid (adults plus 4th and 5th instars) were lower on plants treated with fungus after 30 and 37 days (Fig. 2A). The destructive sampling showed significance among treatments (Fig. 2B; $F_{3,16} = 7.0$, $p = 0.004$). Plants treated with *C. fumosorosea* and plants with *A. herbicolus* plus *C. fumosorosea* showed low densities of *D. citri* and differed significantly from the control (Fig. 2B). The mean number of *D. citri* on plants treated with *A. herbicolus* did not differ from any of the other treatments (Fig. 2B). Number of buds on plants also had a significant effect on the total number of *D. citri* ($F_{3,12} = 4.70$, $p = 0.047$), but the interaction between treatment and number of buds was not significant ($F_{3,12} = 1.0$, $p = 0.42$).

During samplings, we could not confirm the presence of predators through visual inspection of plants and eggs were found on cotton threads from some plants of both treatments with predator a month after the release of mites, but predators were not found on plants nor on black plastic sheets during the destructive counting. During the sampling and the destructive counting, fungi growing on the cadavers of *D. citri* that were exposed to *C. fumosorosea* (Fig. 3) were observed. The fungus was identified as belonging to the *Cordyceps* genus (Fig. 4 and Fig. 5).

DISCUSSION

Concurrent use of a predator with a fungus did not result in a better control of Asian citrus psyllid. Nevertheless, we cannot conclude if the combined action of *A. herbicolus* and *C. fumosorosea* had non-additive effect on the suppression of *D. citri* densities, because not a single mite was found during destructive counting in the treatment with predator plus fungus, neither on plants treated only with predator. Thus, densities of psyllid on plants treated with only *A. herbicolus* did not differ from those on control plants, contrarily to Kalile et al. (2023). Some limitations of our method may explain why predators went extinct on plants.

We think it is unlikely that the absence of *A. herbicolus* on plants was due to direct pathogenicity of *C. fumosorosea*. The pathogen has no detrimental effect on predator biology (Chapter 1). Moreover, predators were also missing from plants that were not treated with *C. fumosorosea*. Spraying plants with conidia suspension or water could have washed away predators from the plants. We found eggs on cotton threads of some plants 23 days after the start of the experiment, although species was not confirmed because threads were discarded. Hence, it is uncertain when mites were extinct from plants. Perhaps mites disappeared from plants at different times, since densities of *D. citri* varied considerably on plants treated with *A. herbicolus*. An experiment in which plants with predators are destructively sampled immediately after spraying water could show whether the mechanical action of spraying removes predators from plants.

Another reason that might have contributed to disappearance of mites on plants is the quality of pollen and frequency it was offered. The pollen was collected and dried in 2019 (Marcossi et al. 2020; Kalile et al. 2021), and it may have lost quality through the years. Besides time, relative humidity and water activity also contribute for deterioration of *Typha* spp. (Buitink et al. 1998). Considering that plants were often sprayed, the higher humidity inside the cages could have fasten degradation of pollen. The decrease of pollen quality could impact *A. herbicolus* fitness on plants. Offering pollen twice a week is sufficient to grow our stock colonies in rearing arenas and this frequency enabled predator persistence in the study of Kalile et al. (2023). Perhaps the same frequency was not enough in our experimental design.

Few other studies investigated the compatibility of predatory mites and entomopathogenic fungi in a dynamics experiment. Direct comparisons with those studies are difficult due to variation of methods and species tested. Some authors also applied fungi on plants more than once, however they used breeding sachets to release hundreds of predators after the fungi treatment application (Wu et al. 2016; Saito and Brownbridge 2018), differing from our study with predator release before application of fungus. We chose to do a single release of predators before releasing *D. citri* because we aimed to assess suitability of *A. herbicolus* as a preventive control strategy, but possibly mites were exposed to higher risk by being released before spraying fungus.

Despite our efforts to standardize plant material, at the end of the experiment plants showed a large variation in height, and consequently, in the number of buds. Young leaves, terminal and axillary buds are important for oviposition and development of *D. citri* (Tsai and Liu 2000); thus, we considered the number of buds in the statistical analyses. We found a correlation between final densities of *D. citri* and the number buds. However, the interaction between treatment and number of buds was not significant, and thus plant variation was not really a problem for final densities of psyllids. It is possible that plant variation could have influenced early establishment of *D. citri*, but we cannot confirm this, because we did not count the number of buds only at the end and not during the experiment. For future experiments, increasing the number of replicates may help mitigate possible effects of plant variation on the results.

As expected, the pathogen was efficient in controlling *D. citri* under greenhouse conditions. Even if mites would have been present until the end of experiment, it would still be possible that the predator combined with the fungus would not give better *D. citri* control than the fungus alone, since such response is expected when a single biocontrol agent is highly efficient in controlling the target pest (Denoth et al. 2002; Straub et al. 2008). The acetate cages used in the experiment contributed to retaining moisture, which created a microclimate inside the cages that was highly favorable for germination and development of fungal spores. The average temperature of the greenhouse close to 26 °C is also optimal for germination of spores and growth of *C. fumosorosea* (Vidal et al. 1997; Borisade and Magan 2014). In other words, the experimental conditions could have biased the efficacy of the fungus treatment. Therefore, studying the combined effect *A. herbicolus* and *C. fumosorosea* in citrus orchards with more variable environmental conditions should provide a more realistic assessment of their concurrent use to control the psyllid.

We attempted to investigate the effects of simultaneous use of *A. herbicolus* and *C. fumosorosea* on the biological control of *D. citri*. Our methodological limitations and the obtained results provide some insights that may be helpful for future research combining fungi and predatory mites.

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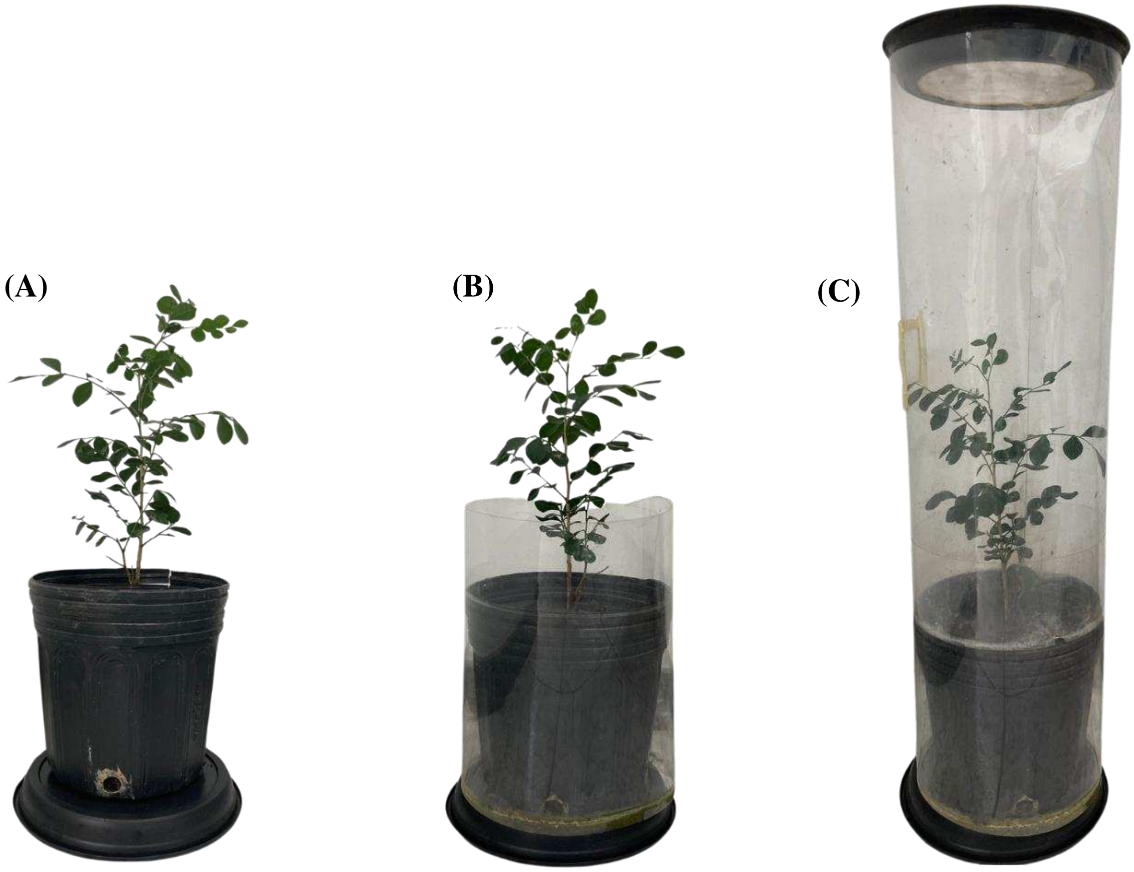

Figure 1: **(A)** Potted plant and saucer. **(B)** Pot surrounded by the first tubular cage. **(C)** Plant surrounded by the second tubular cage.

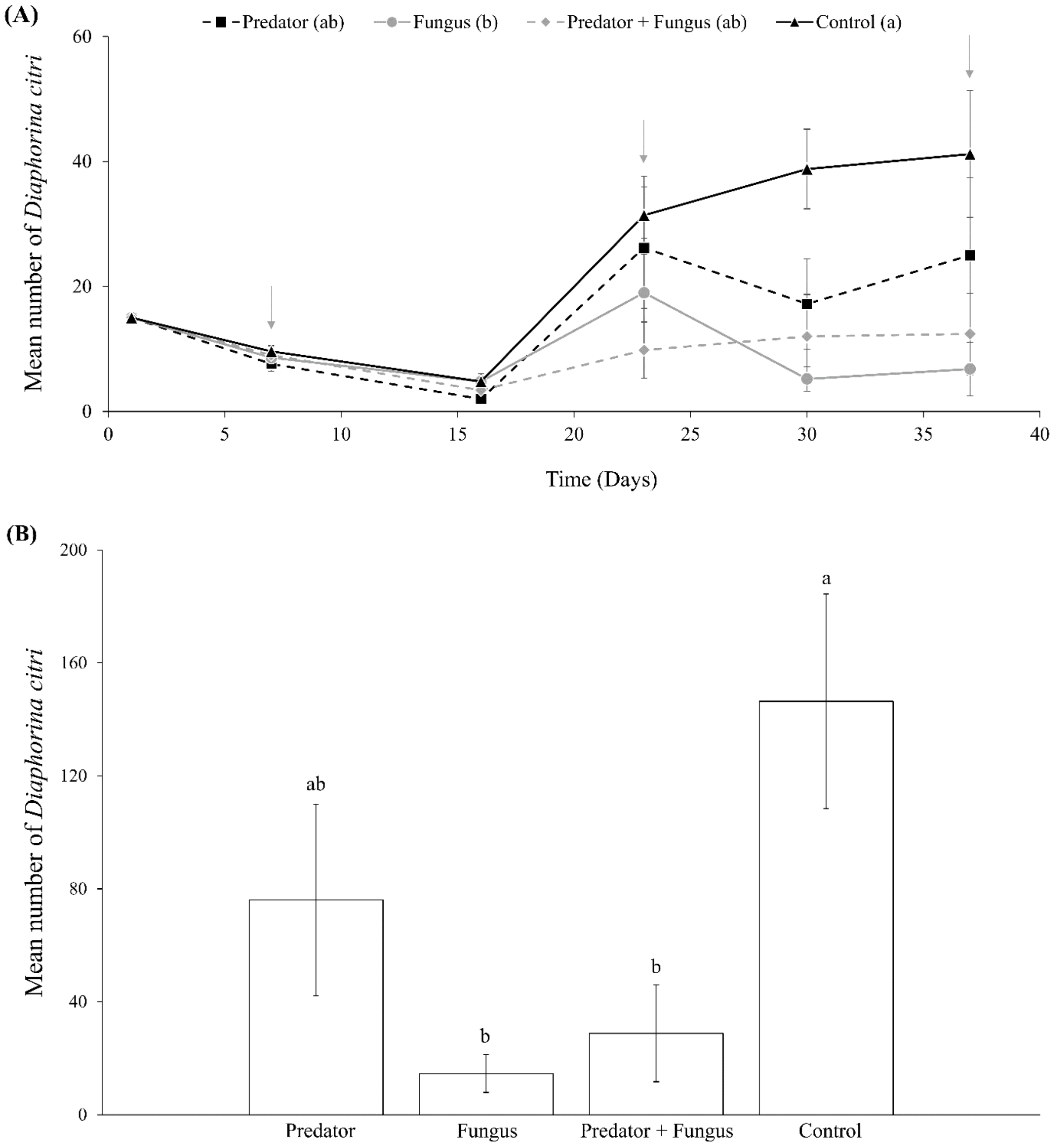

Figure 2: Mean (±SE) number of adults plus fourth and fifth instar nymphs of *Diaphorina citri* on orange jasmine plants exposed to one of: predatory mites, fungal pathogen, combination of predator and fungus or control. Gray arrows indicate when fungi were sprayed. The letters indicate significance of treatments after 30 days (contrasts after LME; $p < 0.05$). **(B)** Mean (±SE) number of *Diaphorina citri* from all life stages at the end of the experiment (destructive sampling on day 44). Bars with different letters indicate significant difference among treatments (contrasts after GLM; $p < 0.05$).

Figure 3: Dead psyllid presenting mycosis during dynamics experiment.

Figure 4: Fungal structure from a dead psyllid growing on PDA medium.

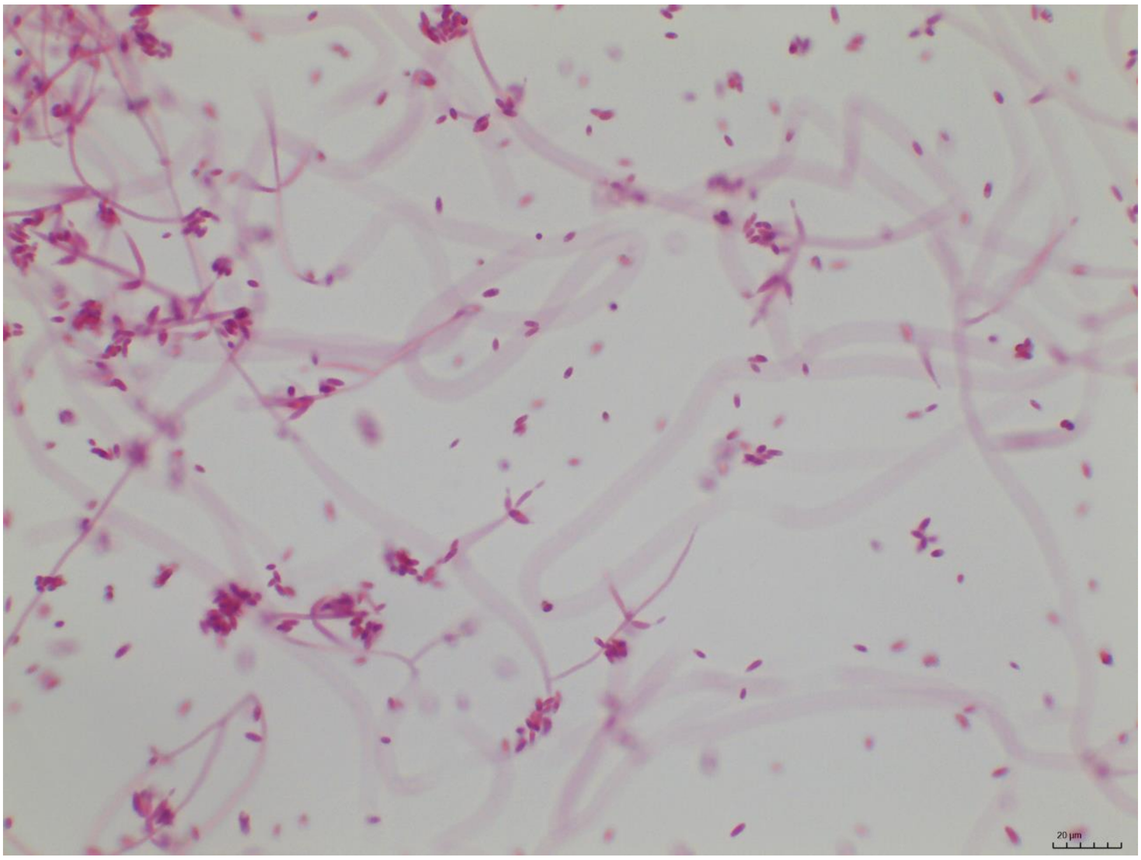

Figure 5: *Cordyceps* spp. conidia and conidiophores (40x magnification).

GENERAL CONCLUSIONS

Adults and juveniles of *A. herbicolus* showed high survival when directly exposed to a conidia suspension of *C. fumosorosea* ESALQ-1296. No adverse effects were observed on juvenile developmental and average oviposition of females. The absence of direct harmful effects of the fungus on the predator suggests that they can be applied together for the biological control of the Asian citrus psyllid.

In a greenhouse study, the combination of *A. herbicolus* and *C. fumosorosea* reduced *D. citri* densities on plants as did the application of fungus alone. Psyllid densities on plants treated with the predator alone were not different from of any other treatment, including a treatment without fungus and predators. It remains unclear whether the concurrent use of the predator and the pathogen enhances the control of *D. citri*, because predators disappeared from the plants during the experiment. Limitations of the methodology were probably responsible for the extinction of predators on the plants.

The obtained results are a first step in the development of an integrated pest management program that combines the releases of predatory mites and application of entomopathogenic fungi with other strategies of control.