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Original Article

Potential of endophytic fungi as biocontrol agents of *Duponchelia fovealis* (Zeller) (Lepidoptera:Crambidae)

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(With 1 figure)

Abstract

This study reports the first assessment of endophytic fungi isolated from strawberry leaves and selection of isolates for the control of *Duponchelia fovealis*, a new pest of strawberries. A total of 400 strawberry leaves of the cultivar 'Albion' were collected in four commercial farms. Leaves were disinfected, cut in fragments, and placed on Petri dishes containing potato dextrose agar media with tetracycline and incubated for 30 days. Following this time, 517 fungal colonies were isolated, and thirteen genera were identified: *Cladosporium*, *Aspergillus*, *Nigrospora*, *Fusarium*, *Trichoderma*, *Chaetomium*, *Alternaria*, *Paecilomyces*, *Penicillium*, *Ulocladium*, *Bipolaris*, *Diaporthe*, and *Phoma.* Eight isolates belonging to the genera *Aspergillus, Diaporthe, Paecilomyces,* and *Cladosporium* were selected for pathogenicity bioassays against third instar larvae of *D. fovealis*. Isolates of *Paecilomyces* induced the highest mortality rates.

Keywords: Fragaria x *ananassa*, European Pepper Moth, microbial control, entomopathogenic fungi.

Fungos endofíticos para o biocontrole de *Duponchelia fovealis* (Zeller) (Lepidoptera: Crambidae)

Resumo

Este trabalho apresenta a primeira contribuição no isolamento de fungos endofíticos de folhas de morangueiro e na seleção de isolados para controle de *Duponchelia fovealis,* uma nova praga do morangueiro. Foram coletadas 400 folhas da cultivar 'Albion' em quatro lavouras comerciais de morangueiro. As folhas foram desinfetadas, cortadas em fragmentos e depositadas em placas de Petri contendo Ágar Batata Dextrose como meio, em conjunto com tetraciclina e incubados durante 30 dias. Um total de 517 colônias fúngicas e treze gêneros foram isolados: *Cladosporium*, *Aspergillus*, *Nigrospora*, *Fusarium*, *Trichoderma*, *Chaetomium*, *Alternaria, Paecilomyces, Penicillium, Ulocladium, Bipolaris, Diaporthe* e *Phoma*. Oito isolados pertencentes aos gêneros *Aspergillus, Diaporthe, Paecilomyces* e *Cladosporium* foram selecionados para os bioensaios de virulência contra larvas de 3º instar de *D. fovealis*. Isolados de *Paecilomyces* causaram as maiores taxas de mortalidade.

Palavras-chave: Fragaria x *ananassa*, *European Pepper Moth*, controle microbiano, fungos entomopatogênicos.

1. Introduction

The European pepper moth, *Duponchelia fovealis* (Zeller, 1847), (Lepidoptera: Crambidae) is native to marshlands of southern Europe and the eastern Mediterranean region (CABI, 2016). This moth is a greenhouse pest of cut flowers, vegetables, and aquatic plants in northern Europe and Canada (CABI, 2016). It has also become a pest of strawberries grown commercially in Europe (Bonsignore and Vacante, 2009; Franco and Baptista, 2010; Efil et al., 2014) and South America (Zawadneak et al., 2016). Because this is an exotic species, arthropod natural enemies are absent or insufficiently effective to control it (CABI, 2016; Zawadneak et al., 2015a). In addition, chemical control may not be as effective as larvae inside stems are protected from treatment (CABI, 2016), requiring several applications. Since strawberries are mostly grown in greenhouses, the control based only on chemical pesticides would result in residues on fruits and impact on the environment (Bernardi et al., 2015). In these cases, microorganisms could be a sustainable alternative control method (Gonzalez et al., 2016) with benefits for

consumers, farmers, and the environment (Bonsignore and Vacante, 2009; Lacey et al., 2015; Gonzalez et al., 2016). For these reasons, biological control based on microorganisms represents a complementary strategy for further development (Zawadneak et al., 2015a; Gonzalez et al., 2016); however, studies on this type of approach involving *D. fovealis* are nonexistent.

Fungi have several characteristics that make them suitable as biological control agents, as they can directly penetrate through the insect cuticle, and increase the potential for epizootics and mortality rates in the pest population (Hajek and Delalibera, 2010; Gonzalez et al., 2016). These fungi can colonize plant tissues as endophytes (Arnold, 2007), apparently remaining assymptomatic (Urrutia et al., 2002; Arnold and Lutzoni, 2007; Vega et al., 2009). Endophytes can have beneficial effects on host plants, e.g., promoting plant growth, reducing disease severity, inducing plant defense mechanisms, and producing anti-herbivore products (Arnold et al., 2003; Arnold and Lewis, 2005; Schulz and Boyle, 2005; Rudgers et al., 2007). These fungi have been detected in hundreds of plant species (Urrutia et al., 2002; Gonzalez et al., 2016).

Endophytic fungi of the genera *Acremonium, Beauveria, Cladosporium, Clonostachys, Paecilomyces, Lecanicillium, Verticillium,* and *Isaria* have been isolated from several cash crops species, such as coffee (*Coffea Arabica* L.), potato (*Solanum tuberosum* L.), corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), tomato (*Lycopersicon esculentum* Mill.), banana (*Musa paradisiaca* L.), date palm (*Phoenix dactylifera* L.), rice (*Oryza sativa* L.), cocoa (*Theobroma cacao* L.), lettuce (*Lactuca sativa* L.). and bean (*Phaseolus vulgaris* L.) (Jones, 1994; Leckie, 2002; Ownley et al., 2004; Posada and Vega, 2005; Quesada-Moraga et al., 2006; Akello et al., 2007; Zawadneak et al., 2015b; Parsa et al., 2016).

This study was aimed at identifying endophytic fungi present in strawberry leaves and select isolates for the control of *Duponchelia fovealis*, a new pest of this crop.

2. Material and Methods

2.1. Samples

Strawberry leaves were collected from four commercial farms in the state of Paraná, Brazil (Table 1), in March 2012, totaling 100 leaves randomly collected per area. Samples were taken to the laboratory in plastic bags and processed on the same day.

2.2. Isolation of endophytic fungi

Leaves were first washed in water to remove dust and dirt residues and then immersed in 70% ethanol for 30 seconds and 1% sodium hypochlorite solution for 3 minutes. The material were then thoroughly rinsed twice in sterile distilled water. After this process, four foliar pieces (2×2cm) were placed in separate Petri dishes (90 × 15 mm) containing potato dextrose agar (PDA) with tetracycline (100 µL/ml). The adaxial part of the leaf was turned upward. Plates were incubated in a BOD chamber at 28 ± 0.5 °C for 30 days. Fungi grown from leaves were transferred to Petri dishes containing PDA media.

The frequency of isolation (FI= number of leaf fragments with fungus growth/ total number leaf fragments) was calculated (Azevedo, 1998).

To compare the frequency of isolation among farms, the data were transformed (log x+1) to normalize the distributions and analyzed with the ANOVA F test and the Tukey test ($p < 0.05$) using the software Assistat 7.7.

2.3. Morphological characterization

The morphological identification of endophytic fungi was based on macro and micromorphology (Kern and Blevins, 1999). Monosporic colonies were cultivated in Sabouraud media and incubated for 14 days at 28 ± 0.5 °C. Non-sporulated isolates were classified as *Mycelia sterilia*. Isolates were maintained at the CMRP (Microbiological Collections of the Parana Network - TAXON line).

2.4. Molecular characterization

2.4.1. DNA isolation and PCR amplification

DNA extraction was carried out according to Badali et al. (2009)

The PCR reaction amplified the regions ITS1-5,8S-ITS2 of ribosomal DNA, partial β-tubulin gene, and partial calmodulin gene with the following pairs of primers: ITS1 5'TCCGTAGGTGAACCTGCGG3' and ITS4 5'TCCTCCGCTTATTGATATGC3' (White et al., 1990), Bt2a 5'GGTAACCAAATCGGTGCTGCTTTC3' and Bt2b 5'ACCCTCAGTGTAGTGACCCTTGGC3' (Glass and Donaldson, 1995), and CL1 5'GARTWCAAGGAGGCCTTCTC3', and CL2A 5'TTTTGCATCATGAGTTGGAC3' (Mulè et al., 2004), respectively.

2.4.2. DNA sequencing

DNA sequencing was performed with 1-3 µL of the purified PCR product, 0.5 µL of buffer, 0.5 µL of each primer, 0.5 µL of BigDye®, and ultra-pure water, making up a final volume of 10 µL. Amplification conditions were: 96 °C for 1 min, and 35 cycles at 96 °C for 10s, 50 °C for 5s and 60 °C for 4 minutes. Sequencing was carried out with a DNA Sequencer Auto Applied BioSystem 3500.

The sequences obtained were aligned and edited with the package Staden Package version 1.6 and the software MEGA version 4.0. Subsequently they were compared

Table 1. Location of the sampling areas of strawberry leaves.

Site	Location	Geographical Coordinates	Crop
A	São José dos Pinhais	25º 36' 753" S and 49º 04' 887" W	Organic
B	Pinhais	25°23'30" S and 49°07'30" W	Organic
C	São José dos Pinhais	25º 33'580" S and 49º 08' 887" W	Conventional
D	São José dos Pinhais	25º 35' 820"S and 49º 05' 866" W	Conventional

with other sequences from the NCBI database with the software BLAST.

2.5. Insects

Larvae used in the experiments were obtained from the rearing facility at the Professor Angelo Moreira da Costa Lima Laboratory of Entomology/ UFPR, fed an artificial diet and kept at 70 ± 10% RH, 25 ± 2 °C, and 14-h photoperiod (Zawadneak et al., 2017).

2.6. Isolated fungi

The endophytic fungi used in the pathogenicity tests were CD72E1 – *Aspergillus terreus*, G13 *Diapothe endophytica*, G28- *Cladosporium cladosporides*, G41- *Paecilomyces lilacinus*, G42- *Paecilomyces lilacinus*, G70- *Aspergillus sydowwi*, OB96E2- *Cladosporium sphaerospermum*, and CD34F1- *Fusarium solani*.

2.7. Fungal preparations

In order to mass produce conidia, isolates were inoculated on malt and incubated at 28 ± 1 °C for 14 days in the dark. Colonies were then scraped and added to 100 mL of sterilized distilled water and Tween 80® (0.001%). The suspension was agitated for ten minutes at room temperature on an orbital shaking incubator at 150 rpm, and filtered through a sterilized cheesecloth. Conidia concentration was determined using a hemocytometer and adjusted to a final concentration of 1×10^9 conidia/mL. Conidial viability was assessed before the suspension preparation, and germination rates were higher than 95% in 24 hours at 28 ± 0.5 °C.

2.8. Pathogenicity bioassay

Eight endophytic fungi were evaluated in bioassays against *D. fovealis*.

Artificial diet was place in twenty-four well culture plates. *D. fovealis* larvae were transferred individually to the wells with the aid of a fine tip brush (#2). This assay consisted of three replicates per treatment and a negative control, totaling 96 larvae per treatment.

In the plate containing 24 larvae, 1mL of conidial suspension at the concentration of 1×10^9 conidia/mL was sprayed with a SAGYMA® Airbrush. A 1mL solution of 0.1% Tween 80 was used as control. Plates were incubated at 25 ± 1 °C, 14-h photoperiod, and 60 ± 10% RH for eight days.

Mortality criteria were based on insect's response to touch, color, and texture. Dead larvae were placed in Petri dishes with moistened sterile cotton for emergence of fungi from the host. The experimental design was completely randomized.

Fungal efficacy was based on means and mortality data (%) (Abbott, 1925) and compared with the ANOVA F test and the Tukey test ($p < 0.05$) with softwares GraphPad Prism 5.03 and Assistat 7.7.

2.9 Estimation of the mean lethal concentration (LC50)

In this second phase of the mortality bioassay, five concentrations were tested 1.0×10^3, 1.0×10^4; 1.0×10^5; 1.0×10^6; 1.0×10^7 and 1.0×10^8 conidia/mL of the isolates that induced the highest mortality rates in the pathogenicity bioassay.

The procedures were the same as those used in the first phase, consisted of *D. fovealis* larvae in twenty-four well culture plates sprayed with a conidial suspension at a given concentration, with five replicates per treatment. Mortality was assessed using the same criteria described previously.

The experimental design was completely randomized and the results were compared with the Probit analsyis) with Polo-PC – Leora Software, 1987.

3. Results

3.1. Prevalence of isolates

A total of 1600 foliar fragments were collected from the four areas and 517 endophytic colonies were isolated. The prevalence of isolates was 32% (Table 2), varying among the areas evaluated, with values ranging from low to average prevalence (24.3 - 45.8%). Area A showed the highest number of isolates (183), followed by area B with 123 isolates, while areas C and D had 114 and 97 ($F: 13.2939$, df: 3; 396; $p < 0.05$) isolates, respectively.

3.2. Composition of the endophytic assemblages

The 278 endophytic fungi isolated in this study were categorized into 15 taxa (Table 3) based on morphological characteristics, but 239 non-sporulating fungi could not be identified.

The genera *Aspergillus* spp., *Paecilomyces* spp., *Alternaria* spp., *Penicillium* spp. and *Cladosporium* spp. were separated into two or more morphospecies based on the size of the colony and conidial characteristics. As shown in Figure 1, *Cladosporium* sp. had the highest frequency of isolates. *Alternaria* sp. was the second most frequent endophytic taxon followed by *Diaporthe* sp., with frequencies of 19, 12, and 9.31% respectively.

The fungi used in the tests against *D. fovealis* caterpillars were identified by molecular analysis as: *Aspergillus terreus* (CD72E1), *Aspergillus sydowwi* (G70), *Paecilomyces lilacinus* (G41; G42), *Cladosporium sphaerospermum* (OB96E2), *Cladosporium cladosporides* (G28), *Diaporthe endophytica* (G13), and *Fusarium solani* (CD34F1). Sequencing of

Table 2. Prevalence of isolates and number of taxa of fungal endophytes isolated from strawberry leaves at each site.

Characteristic	Site A	Site B	Site C	Site D
Nº of samples	100	100	100	100
Nº of isolates	183 *a	123 *b	114 *b	97 *b
average deviation	1.146	0.941	0.829	0.926
Isolate prevalence (%) **	45.8%	30.7%	28.5%	24.3%

* Comparison of means using the Tukey test at 0.05 probability. Same lowercase letters do not differ at 95% level; ** Isolate prevalence (%) = (total number of leaf pieces yielding more than one isolate)/(total number of leaf pieces in that trial) x100.

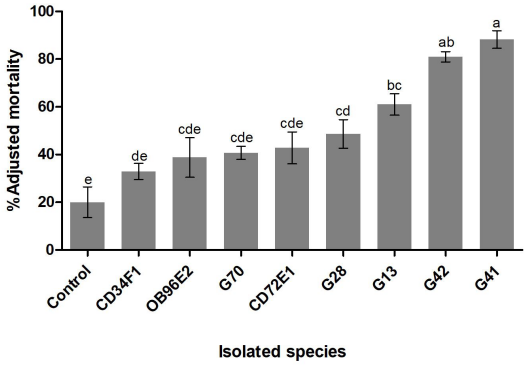

Figure 1. Adjusted mortality percentage induced by conidia suspension and distilled water (control). Tukey test was significant at 1% (F 23.64 = *df* = 8). CD34F1 = *Fusarium solani*; OB96E2 = *Cladosporium sphaerospermum*; G70 = *Aspergillus sydowwi*; CD72E1 = *Aspergillus terreus*; G28 = *Cladosporium cladosporides*; G13 = *Diaporthe endophytica*; G42 = *Paecilomyces lilacinus*; G41 = *Paecilomyces lilacinus*.

Table 3. Prevalence of endophytic fungal genus isolated from strawberry leaves.

Genera	Number of isolates	%
Trichoderma sp.	2	0.7
Fusarium spp.	2	0.7
Paecilomyces spp.	4	1.43
Nigrospora sp.	11	3.95
Ulocladium sp.	15	5.39
Diaporthe sp.	16	5.75
Phyllosticta sp.	16	5.75
Bipolaris sp.	17	6.11
Phoma sp.	18	6.47
Chaetomium sp.	18	6.47
Penicillium sp.	21	7.55
Mycelia sterilia	24	8.63
Aspergillus spp.	27	9.71
Alternaria spp.	39	14.02
Cladosporium spp.	48	17.26
**Unidentified*	239	-

* Non-sporulated monosporic colonies.

fungi re-isolated from dead larvae at the end of each test confirmed the presence of the species used in the experiment (Table 4).

3.3. Pathogenicity assay

The results showed that treatments with fungi had a significant effect (F 23.64 = df 8 $p < 0.01$) on the mortality of larvae, ranging from 32 to 88%, compared to 20% for the negative control (Figure 1). Isolates could be categorized into three groups: isolates that induced low mortality (>40%), intermediate mortality (45–61%), and highly mortality rates (> 80%). The treatment with the lowest performance was isolated CD34F1 (*F. solani*) with 32% mortality against *D. fovealis*. Isolates G70 (*A. sydowii*) and CD72E1 (*A. terreus*) induced mortality rates of 40 and 42%, respectively; while for isolates OB96E2 (*C. sphaerospermum*) and G28 (*C. cladosporioides*), mortality rates were 38 and 48%, respectively. Isolate G13, identified as *D. endophytica*, induced mortality of nearly 61% of larvae. The most virulent isolates obtained from mortality bioassays were G42 (*P. lilacinus*) and G41 (*P. lilacinus*) which induced mortality rates of 80 and 88%, respectively.

3.4. Estimation of the mean lethal concentration (LC50)

The values of LC50 of two *P. lilacinus* isolates (G41 and G42) were similar (Table 5). The low slope (0.524 ± 0.081 and 0.422 ± 0.050) and $\chi 2$ values (0.444 and 0.880), respectively indicate the Probit model was a good fit to the data. The effective lethal concentration was 3.6×10^7conidia/mL for G41 and 5.4×10^7 conidia/mL for G42.

4. Discussion

The highest number of fungal endophytes isolates was obtained from strawberry leaves collected in area A, which has been an organic farm for 12 years. Although area B is also characterized as an organic grower, the period of time converting from the conventional system to organic production was only three years and the biodiversity found was similar to those of conventional areas. The model of conventional agricultural is characterized by single crops, large dependence on chemical products, and high yields (Feiden et al., 2002). A considerable biodiversity is lost

Table 4. Molecular identification of species used in the mortality bioassay against *D. fovealis*.

Species	Strain	GenBank Number	Gene
Aspergillus terreus	CD72E1	KT427380	β-Tubulin
		KT427386	Calmodulin
Aspergillus sydowwi	G70	KT427381	β-Tubulin
Paecilomyces lilacinus	G41	KT427382	β-Tubulin
Paecilomyces lilacinus	G42	KT427383	β-Tubulin
Diaporthe endophytica	G13	KT427385	ITS
		KP2164961	Calmodulin
Cladosporium sphaerospermum	OB96E2	KP2164981	ITS
Cladosporium cladosporides	G28	KP2164971	ITS
Fusarium solani	CD34F1	KP2164951	ITS

Table 5. Probit analysis of mortality and log-concentration of conidia using in the bioassay with two isolates against third instar larvae of *D. fovealis*.

Treatment	G41	G42
Nº of larvae	689	671
[a] LC_{50}	3.6×10^7	5.4×10^7
Slope(SE)	0.524 ± 0.081	0.422 ± 0.050
95% FL	1.2×10^7- 8.4×10^7	1.3×10^7 - 9.2×10^7
χ^2 (df=4)	0.444	0.880

[a] LC50 values and their 95% confidence levels are expressed in conidia per milliliter.

before being known, due to habitat fragmentation, excessive exploitation of natural resources, and contamination of soil and water, as well as of organisms in the agroecosystem (Altieri et al., 2005). Thus, information on the biodiversity is an important strategy in the ecological management of the populations inhabiting in the agroecosystem. Biodiversity creates a balance among species that, when it is restored, numerous and complex interactions are established among soil, plants, and animals (Altieri et al., 2005).

The genera *Cladosporium*, *Trichoderma*, *Diaporthe*, *Phoma*, and *Alternaria* isolated in this study can be used as antagonistic agents against phytopathogenic fungi. *Cladosporium* sp., *Aspergillus* sp., *Nigrospora* sp., *Fusarium* p., *Trichoderma* sp., *Chaetomium* sp., *Alternaria* sp., *Paecilomyces* sp., and *Phyllostica* are often isolated as endophytic fungi in several agricultural and native plant species (Azevedo, 1998; Rubini et al., 2005; Guo et al., 2008; Veja et al., 2008; Gazis and Chaverri, 2010; Kurose et al., 2012; Parsa et al., 2016). The genus *Trichoderma* sp. has been shown to induce resistance and enable plant defense (Rubini et al., 2005; Verma et al., 2007; Bailey et al., 2009; Kurose et al., 2012), while some species of the genera *Fusarium* and *Paecilomyces* can be used to control plant parasitic nematodes (Mendoza and Sikora, 2009). Furthermore, *Aspergillus* spp., *Penicillium* spp., and *Chaetomium* sp. are used in the industry for the production of secondary metabolites that exhibit antimicrobial and antifungal properties (Hanada et al., 2010; Li et al., 2011; Wang et al., 2012; Jouda et al., 2014).

C. cladosporioides and *C. sphaerospermum* were pathogenic for *D. fovealis larvae.* Endophytic species of the genera *Cladosporium* are used in the biocontrol of pests and diseases (Veja et al., 2008). Bahar et al. (2011) tested a strain of *Cladosporium* against larvae of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) and reported increased mortality rates of larvae. Similar results were obtained in this study, where *C. cladosporioides* and *C. sphaerospermum* were pathogenic to *D. fovealis* larvae.

Among the tested fungi, the isolates of *P. lilacinus* induced a high mortality rate of *D. fovealis* larvae. *P. lilacinus* is widely used in the biological control of diseases in plants cultivated under field conditions (Veja et al., 2008). Other studies performed with this species reported mortality in nymphs of *Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae) (Fiedler and Sosnowska, 2007) and showed a potential in the control of *Nasonovia ribisnigri* (Mosley, 1841) (Hemiptera, Aphididae) a pest of lettuce (*Lactuca sativa* L.) in Brazil (Zawadneak et., al. 2015b) and its applicability in the control of nematodes of the genus *Meloidogyne* sp. (Fiedler and Sosnowska, 2007).

In the present study, the isolate *D. endophytica* induced a mortality rate of 68% in *D. fovealis*. This endophytic species has not been reported as entomopathogenic in the literature. This is the first study to report this genus as a potential biological control agent of insects.

The isolates G41 and G42 had LC50 values considered good compared to those reported in the literature. Other studies have found significant results for isolates of this genus, as observed in our study. Hussein et al. (2013) treated third instar larvae of *Spodoptera littoralis* (Noctuide: Lepidoptera) with a new lineage of *Paecilomyces* sp (CCM 8367) in a suspension at a concentration of 5.0×10^7 conidia/mL and reported a mortality rate above 90%.

In conclusion, G41 and G42 isolates induced the highest mortality rates in *D. fovealis* larvae with the lowest LC50. Our findings showed the pathogenic and virulence potential of *Paecilomyces lilacinus* isolates as biological control agents against *D. fovealis* in laboratory as an alternative to currently employed traditional chemical insecticides. This is the first study on endophytic fungi isolated from strawberry tested against *D. fovealis*.

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