FACETS
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RESEARCH ARTICLE

Orbitides and free polyamines have similarly limited fungicidal activity against three common pathogens of flax *in vitro*

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OPEN ACCESS

Citation: Alves da Costa Ribeiro Quintans IL, Alves da Costa Ribeiro Souza J, and Deyholos MK. 2022. Orbitides and free polyamines have similarly limited fungicidal activity against three common pathogens of flax *in vitro*. FACETS 7: 843–852. doi:10.1139/facets-2021-0201

Handling Editor: Allison Kathleen Walker

Received: December 18, 2021

Accepted: May 2, 2022

Published: June 9, 2022

Note: This paper is part of a collection titled "The Canadian Fungal Network 2021 Annual Meeting – Connecting fungal researchers across Canada".

Published by: Canadian Science Publishing

Abstract

Fusarium oxysporum f. sp. *lini* and *Septoria linicola* are causes of fusarium wilt and pasmo in flax (*Linum usitatissimum*). Members of a third fungal genus, *Alternaria* spp., have also been found in fiber and linseed varieties of flax, and are a source of post-harvest spoilage and mycotoxins in a wide range of crops. We performed a microdilution assay and calculated the median effective concentration (EC_{50}) to compare the potency of cyclolinopeptides (CLPs), two polyamines (spermidine and spermine), and the fungicide carbendazimin in the control of three fungi that have potential pathogenic activity (*F. oxysporum*, *S. linicola*, and *Alternaria* spp), of which the first two are particulary significant causes of disease in flax. For carbendazim, all EC_{50} values were <0.6 µg/mL. The observed EC_{50} ranged from 111 to 340 µg/mL for a mixture of six unique CLPs, 109 to 778 µg/mL for spermine, and 21 to 272 µg/mL for spermidine. Spermidine was most effective against *Alternaria* sp., with an EC_{50} of 21 µg/mL. The results presented here showed that polyamines and CLPs possess limited antifungal activities against several fungi, with spermidines the most effective naturally occurring compound tested. Our findings do not support the hypothesis that CLPs act as potent antifungals against the three species of pathogens tested.

Key words: CLPs, antifungal assay, polyamines, orbitides, phytopathogens, fungi, microdilution, inhibitory

Introduction

The fungi *Fusarium oxysporum* f. sp. *lini* and *Septoria linicola* are causes of fusarium wilt and pasmo, which are two commercially significant diseases of flax (*Linum usitatissimum*). *Alternaria* spp., have also been found to be very prevalent in harvested flax material and can cause destructive diseases in various plants (Mankevičienė et al. 2006). Agricultural measures for crop protection include the use of chemicals, resistant plant varieties, biological control, and modification of agricultural practices (Ribeiro-Quitans 2019). We propose to test members of two classes of flax endogenous compounds, CLPs and PAs, for their potency against pathogens of flax. This information may help identify future breeding targets or plant protection strategies.

Flax seeds are one of the richest sources of CLPs, which are N-to-C cyclized amino acids (Burnett et al. 2016). Several nutraceutical benefits have been proposed for CLPs, involving antiplatelet, antimalarial, antioxidant, and immunomodulatory activities (Sharav et al. 2014; Tan and Zhou 2006). Furthermore, the high thermostability of CLPs may be beneficial in some industrial applications, for example to withstand postharvest heat treatments applied to control insects and microbes (Lurie and Pedreschi 2014). Despite the range of health and industrial applications under development, the natural biological function of CLPs in plants is unknown; although, a role for these peptides in plant defense has been proposed (Arnison et al. 2013). For instance, transcripts encoding the precursors of CLP #5 ([1—8-NαC]-linusorb A2) and CLP #14 ([1—8-NαC]-linusorb A1) increased up to 4.7-fold in abundance upon exposure of flax roots to *F. oxysporum* (Burnett et al. 2016). Furthermore, flax CLPs inhibited spore formation by other pathogenic fungi, *Aspergillus flavus* (IC_{50} 240 µg/mL) and *Paecilomyces varioti bainier* (IC_{50} 340 µg/mL) (Zun et al. 2017).

On the other hand, PAs are natural plant endogenous compounds, with well-known antifungal properties (Takahashi and Kakehi 2010; Wojtasik et al. 2015). Polyamines are linear, aliphatic amines, including spermidine (a triamine) and spermine (a tetraamine) (Wojtasik et al. 2015). Exogenous application of spermines to plants induced defenses against several pathogens, mainly through the activation of the plant immune system (Seifi and Shelp 2019). It has been previously shown that there is a great increase in the abundance of PA-related transcripts in flax as a response to *F. oxysporum* (Wojtasik et al. 2015; Ribeiro-Quitans 2019). Additionally, it has been reported that presence of PAs in solid agar medium restricts *Fusarium* growth in a dose-dependent manner; statistically significant reductions in the radius of hyphal growth were observed with a minimum of 10 mM (880 µg/mL) putrescine, 6 mM spermidine (871.5 µg/mL), and 3 mM (607.02 µg/mL) spermine (Wojtasik et al. 2015). It should also be noted that the exogenous application of polyamines to plants can disrupt normal metabolic and developmental processes (e.g., pollen germination and elongation), leading to toxic effects (Wolukau et al. 2004; Setia and Setia 2018; Sorkheh et al. 2011).

To investigate whether CLPs could inhibit fungal growth *in vitro*, we obtained a commercially available mixture of six unique CLP and performed a microdilution assay to test the activity of this CLP mixture against three potential plant pathogens (*F. oxysporum*, *S. linicola*, and *Alternaria* sp.). We compared the efficacy of the CLP mixture to carbendazim, a broad-spectrum commercial fungicide, and two PAs, spermidine and spermine. We chose these PAs to represent low molecular weight, endogenous compounds that have previously been shown to inhibit fungal growth.

Material and Methods

Fungal sources and culture

Fusarium oxysporum f.sp. *lini* isolates 13 and 81 were generously provided on potato dextrose agar (PDA) by Dr. Khalid Rashid (Agriculture and Agri-Food Canada, Morden, MB, Canada) and Dr. Martin Reaney (University of Saskatchewan), respectively. Dr. Reaney also provided *Septoria linicola* isolate (14SL43) in the same conditions. *Alternaria* sp. isolate (BH 503) was provided by Dr. Louise Nelson (University of British Columbia). It should be noted that there has been no controlled assessment of the pathogeneticy of isolate BH 503.

Cultures were grown on PDA at room temperature in the dark until growth covered the plates (approximately 14 days). Spores were isolated by flooding the plate with sterile water with 1% Tween 20 and gently rubbing with a sterilized loop. The solution was filtered using a sterile

cheesecloth to isolate the spores and remove the mycelia. Spore concentration was quantified using a hemocytometer to the required final concentration of 10^5 spores in each well of the microdilution assay.

Test compound sources and dilutions

A CLP mixture extracted from *L. usitatissimum* was kindly provided as a lyophilized powder by Martin Reaney (Prairie Tide Diversified Inc.; CLMIX, Lot M201304-001). The CLPs were tested as a mixture because this product is commercially available, is representative of the most abundant CLPs in flax, and pure isolates of individual CLPs are prohibitively expensive. The composition of this powder is listed in Table 1, and the composition-weighted mean molecular mass of the mixture is 1051.6 g/mol. Other test compounds were purchased: carbendazim (Sigma 378674, 97% pure, 191.19 g/mol, CAS 10605-21-7), spermidine (VWR CAAAA19096-06, 99% pure, 145.25 g/mol, CAS 124-20-9), and spermine (VWR CAAAAL19562-06, 97% pure, 202.35 g/mol, CAS 71-44-3). *In vitro* inhibitory assays for of the CLP mixture and carbendazim followed the standard broth dilution method (EUCAST 2019), with a few modifications. Briefly, the CLP mixture and carbendazim were dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) at 200× the desired final concentration. A two-fold dilution series was prepared in DMSO, with 10 different concentrations. Subsequently, another dilution was performed by taking 100 µL of each tube with 200× concentration of the substance to be tested and transferring this to 9.9 mL of malt extract 2% medium (VWR, 97063-426) (1:100 dilution). This reduced the concentration of the solvent in the culture tubes to 1% and the concentration of the tested compounds to 2×. For experiments on the effect of PAs, the highest concentration was prepared at 5× the final concentration in sterile water. A two-fold dilution series was prepared from that in water, with 10 different concentrations.

Microdilution assays were performed in sterile 96-well polystyrene plates (Greiner Bio-One, Monroe, NC). The CLP mixture concentration ranged from 0 to 512 µg/mL and the concentration of carbendazim ranged from 0 to 50 µg/mL. For the CLP mixture and carbendazim assays, each plate was prepared by adding 100 µL of the 2× final concentration of the compound in 2% malt extract into the wells of the columns 1 to 10. For example, for the CLP mixture, we dispensed to each well in column 1 the medium containing 512 µg/mL of CLP mixture, 256 µg/mL to column 2, 128 µg/mL to column 3, and so forth. Wells from the columns 11 and 12 had only the media, without the tested compounds.

The concentrations for the PA assay were chosen based on a previously published study (Wojtasik et al. 2015). The highest concentration for the 2× dilution was 10 mM for each PA, which corresponds to 1452 µg/mL of spermidine and 2023 µg/mL of spermine. Each plate was prepared by adding 20 µL

Table 1. Composition of CLP mixture.

Orbitide name	Proportion of total	CAS #	g/mol
CLA	22.22%	33302-55-5	1040.34
CLB	20.63%	222527-65-3	1074.38
CLD	7.94%	222527-66-4	1064.34
CLE	20.63%	222527-67-5	977.26
CLF	7.94%	351417-15-6	1084.35
CLG	20.63%	351417-16-8	1098.38

Note: For calculation of molecular mass, the sulfoximine forms of each cyclolinopeptides (CLP) was used, where applicable.

of the 5× final concentration of the compound in water into the wells of the columns 1 to 10. Wells from columns 11 and 12 had only 20 µL of water, without the compounds.

Fungal growth inhibition assay

For the CLP mixture and carbendazim, we added 100 µL of the spores suspension to each well of the plate (except for the column 12, where it was the negative control) to achieve the final concentration of 1×10^5/mL, as recommended by EUCAST (2019); 100 µL of the sterile DI water used to prepare the spore dilution was added to column 12. The *in vitro* inhibitory assays of PAs were performed according to Zeitler et al. (2013). An 80 µL aliquot of fungal spores at 10^5 spores/mL resuspended in 2% malt extract media was added per well resulting in final fungi concentration of 1×10^5/mL spores and a final compound concentration of 0–10 mM of each polyamine, which corresponds to: spermidine (0–1452 µg/mL) and spermine (0–2023 µg/mL). We added 100 µL of the water used to prepare the spore dilution to column 12.

An initial absorbance reading at λ = 530 nm was recorded for all the plates using the Varioskan LUX Multimode Microplate Reader. After incubation for 2 days at room temperature in the dark (and 3 days for *S. linicola*), fungal growth was determined by measuring optical density (OD_{530nm}) with the Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA) and by visual inspection.

The median effective concentration (EC_{50}) was calculated as the concentration at the midpoint between the baseline growth rate and the dose point at which maximum inhibition occurred. The EC_{50} values were calculated using the drc package in R (Ritz et al. 2015). We performed at least two biological independent experiments for each assay, with eight technical replicates per experiment, for a total of 16 technical replicates across all experiments.

Results

Measurement of EC_{50} in a fungal inhibition assay

To test whether a mixture of CLPs could inhibit growth of plant pathogenic fungi *in vitro*, we conducted a microplate assay in which wells of a 96-well plate were inoculated with fungal spores in malt extract broth, with a two-fold dilution series of a CLP mixture extracted from flax seeds as well as controls that lacked the CLP mixture. Fungal growth was measured by absorbance at 530 nm. In parallel to the CLP mixture, we also tested three compounds as positive controls: a commercial synthetic fungicide (carbendazim) and two polyamines (spermidine and spermine). We applied this method to three phytopathogenic fungi (*F. oxysporum*, *S. linicola*, and *Alternaria* sp.). Two different isolates of *F. oxysporum* were tested. The results of this growth inhibition assay are shown in **Fig. 1**. We observed that the CLP mixture and both PAs caused dose-dependent decreases in growth of all of the fungi tested. Carbendazim also decreased growth of *F. oxysporum* and *S. linicola* but did not have any detectable negative effect on *Alternaria* sp. growth.

To quantify the potency of each inhibitory compound, we used the drc package of the R statistical computing environment (Ritz et al. 2015) to fit a dose-response curve, from which an EC_{50} value and standard errors were calculated (**Tables 2** and **3**). The EC_{50} value is the concentration at which fungal growth was reduced to 50% of the control based on the fitted curve. The EC_{50} ranged from 111 to 340 µg/mL for the CLP mixture, 21 to 272 µg/mL for spermidine, 109 to 778 µg/mL for spermine, and the EC_{50} for carbendazim was <0.6 µg/mL (**Table 2**).

No EC_{50} could be calculated for *S. linicola* with carbendazim, because the EC_{50} in this case was lower than the minimum concentration tested. Conversely, no EC_{50} could be calculated for *F. oxysporum*

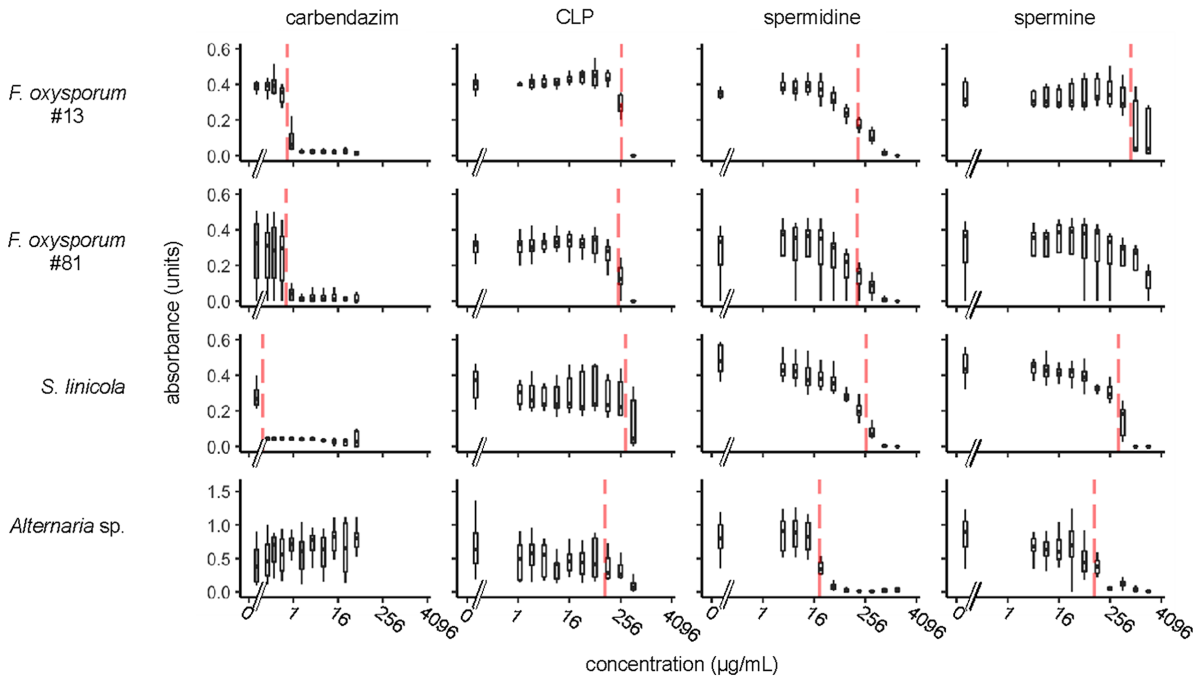

Figure 1. Growth of fungi in the presence of varying concentrations of potential inhibitors. Growth is represented as A_{530} absorbance. Data shown for each fungus and concentration represent minimum of 16 technical replicates in two independent experiments. Box-and-whisker plots show the median (black bar) surrounded by a box spanning the interquartile range (IQR, 25th to 75th percentile) of all observations. Whiskers extend to the largest and smallest observations that are not more than 1.5× the IQR from the median. Where a vertical red dashed line is present, it represents the EC_{50}. The *x*-axis is in log-scale, and thus the "0" concentration of inhibitor is represented by a discontinuity.

#81 with spermine, presumably because full inhibition of growth was not achieved at the highest concentration tested, and thus an appropriate curve could not be fit.

For *S. linicola* and both isolates of *F. oxysporum*, carbendazim was the most effective antifungal agent, usually by two to three orders of magnitude, when its EC_{50} value was compared to the CLP mixture or either of the PAs (Tables 2 and 3). Conversely, spermine was consistently the least potent growth inhibitor tested with these particular fungal species. The CLP mixture and spermidine were roughly comparable in EC_{50} values, and the CLP mixture was either slightly more or less potent than spermidine depending whether concentration was considered as mass per volume (Table 2) or as molarity (Table 3).

When *Alternaria* sp. was tested, we found that carbendazim had no detectable inhibitory effect. However, *Alternaria* sp. was more sensitive to the three other test compounds (CLP mixture, PAs) than were *F. oxysporum* or *S. linicola*. Spermidine appeared very effective against *Alternaria* sp., with an EC_{50} of 21.14 µg/mL.

Discussion

The natural biological roles of orbitides (i.e., CLPs) are not well established. Because of the hydrophobic composition of CLPs, it has been suggested that they may have a membrane-active role, possibly resembling antimicrobial peptides (AMPs) (Arnison et al. 2013; Arouri et al. 2011). However, CLPs differ from AMPs, because the CLPs identified so far are not cationic. Therefore, we were motivated to test whether a mixture of CLPs isolated from *L. usitatissimum* could inhibit growth of three fungi

Table 2. Summary of EC_{50} from fungal growth inhibition assays (mass/volume basis).

	Carbendazim (µg/mL)		CLP (µg/mL)		Spermidine (µg/mL)		Spermine (µg/mL)	
	EC_{50}	se	EC_{50}	se	EC_{50}	se	EC_{50}	se
Fusarium oxysporum #13	0.580	0.018	269	39.0	171	11.7	778	127
F. oxysporum #81	0.540	0.064	230	14.5	164	42.7	—	0
Septoria linicola	< 0.5	—	340	899	272	117	406	42.0
Alternaria sp.	—	—	111	126	21.0	0.900	109	15.0

Note: EC_{50}, median effective concentration; CLP, cyclolinopeptides; se, standard error.

Table 3. Summary of EC_{50} from fungal growth inhibition assays (molar basis).

	Carbendazim (µM)		CLP (µM)		Spermidine (µM)		Spermine (µM)	
	EC_{50}	se	EC_{50}	se	EC_{50}	se	EC_{50}	se
Fusarium oxysporum #13	3.03	0.09	255.8	37.09	1177	80.55	3845	627.6
F. oxysporum #81	2.82	0.33	218.7	13.79	1129	294.0	—	0
Septoria linicola	—	—	323.3	854.9	1872	805.5	2006	207.6
Alternaria sp.	—	—	105.6	119.8	144.6	6.2	539	74.13

Note: EC_{50}, median effective concentration; CLP, cyclolinopeptides; se, standard error.

commonly associated with plants: *F. oxysporum*, *S. linicola*, and *Alternaria* sp. Of these, *F. oxysporum* and *S. linicola* are significant pathogens of flax, but the pathogenicity of this local *Alternaria* sp. isolate has not been established. For comparison, we also assayed a commercial fungicide (carbendazim) and two PAs that have previously been shown to inhibit growth of some of these fungi in other types of assays (Amini and Sidovich 2010; Wojtasik et al. 2015). We found that the median effective concentration (EC_{50}) for the CLP mixture in our growth inhibition assays ranged from 111 to 340 µg/mL (**Table 2**). These results are consistent with the EC_{50} ranges reported by Zun et al. (2017) for CLPs with two other fungi. In our study, for each fungus assayed, EC_{50} values were slightly higher for the CLP mixture than for spermidine (21–272 µg/mL) and lower or similar for the CLP mixture than spermine (109–778 µg/mL). In contrast, EC_{50} values for carbendazim for *F. oxysporum* and *S. linicola* (<0.58 µg/mL) were much lower than the CLP mixture or PAs. Thus, we conclude that both the CLP mixture and PAs have measurable fungicidal activity, but their potency against *F. oxysporum* and *S. linicola* is weak when compared to a commercial fungicide.

Alternaria spp., are known to be resistant to benzimidazole fungicides such as carbendazim, and this was consistent with our observations (Eckert and Ogawa 1985). However, spermidine was more effective against *Alternaria* sp. (EC_{50} 21 µg/mL) than either of the other fungi we tested. These results raise an interesting question: Is the efficacy of spermidine on *Alternaria* sp. related the necrotrophic lifestyle of the fungus? It has been suggested that there are differences on PA biosynthesis induced by either biotrophic or necrotrophic pathogens in plants (Pal and Janda 2017); however, the role of PA metabolism in the interaction of plants with pathogens with different lifestyles is still not clear. Further work is required to establish the role of free spermidines on the growth inhibition of necrotrophic pathogens, such as *Alternaria* spp. It must further be emphasized that although the isolate used

in these experiments were obtained from diseased plant tissue, the pathogenicity of this isolate has not been established. Therefore, there are potentially many explanations for the observed sensitivity of this isolate to spermidine. Furthermore, any potential applications of exogenous PAs as fungicides must take into consideration both the established and potentially still unknown toxic effects of PA application on plants (Wolukau et al. 2004; Setia and Setia 2018; Sorkheh et al. 2011).

Although for both *F. oxysporum* and *S. linicola*, all of the natural compounds we tested were orders of magnitude weaker as fungicides than carbendazim, the effective ranges we observed for some of these natural compounds are near to what might be present *in planta*, under some conditions. For example, the concentration of free spermidine in flax seedlings exposed to *F. oxysporum* is 10 µg/g of fresh weight, with the inclusion of conjugated and bound forms of spermidine this increases to 65 µg/g of fresh weight (Wojtasik et al. 2015). The EC_{50} we observed for spermidine ranged from 21 to 272 µg/mL, and suppression of fungal growth was evident far below the median (**Fig. 1**). Therefore, we cannot yet exclude the possibility that spermidine has some antifungal activity in the phyllosphere, rhizosphere, or within the plant itself. Furthermore, our results show that free spermidine can inhibit fungal growth, even in the absence of conjugation to other plant-produced molecules or induction of plant signaling cascades. Thus spermidine may have a direct role in modulating fungal growth, in addition to its many other roles in defense and other processes, both within fungi and within plants (Jiménez-Bremont et al. 2014; Pal and Janda 2017). On the other hand, spermine was less potent than spermidine in our assays. Moreover, endogenous spermine is less abundant in flax seedlings than spermidine. Thus, the reported ability of spermine to promote resistance to disease may be due to the modulation of the plant immune system rather than direct fungicidal activity (Seifi and Shelp 2019; Takahashi 2016).

Flax is one of the richest natural sources of CLPs, and the concentration of CLPs in flax seeds can be as high as 300 µg/g (Arnison et al. 2013; Gui et al. 2012). Concentrations of CLPs are likely much lower in roots, but it is possible that CLP abundance could be increased in response to a pathogen *in vivo*. It also remains possible that CLPs could act more effectively as fungicides *in planta*, as part of multicomponent defenses. This is consistent with the ideas of Fisher et al. (2018), who suggested that PawL-derived Peptides, orbitides from the subfamily Asteroideae, might have antibacterial effects *in vivo* working as multicomponent antimicrobials (Fisher et al. 2018; Garneau et al. 2002). Further studies on the current topic are therefore recommended, to elucidate synergistic effects of CLPs *in vivo*.

Conclusion

F. oxysporum, *S. linicola*, and *Alternaria* sp. were each affected by at least one of the compounds studied (polyamines, carbendazim, and a mixture of CLPs), where spermidines were the most effective natural occurring compound against phytopathogens, especially *Alternaria* sp, on a mass/volume basis, and the CLP mixture was most effective on a molar basis. Conversely, carbendazim application did not inhibit the growth of *Alternaria* sp., whereas this fungicide greatly affected the growth of *F. oxysporum* and *S. linicola in vitro*. Although the CLP mixture and spermidine were much less potent than carbendazim, both the CLP mixture and spermidine showed fungicidal activity within a concentration range that might be biologically relevant. Moreover, these results show that a CLP mixture and spermidine can have direct effect on fungal growth, independent of any other contributions from the plant host. The findings reported here shed new light on the participation of CLPs in the protection of plants against pathogens in plants, and as well the inhibitory effects of free spermidines on fungi, especially those with a necrotrophic lifestyle. These results open new opportunities for studies to test the effects of exogenous applications of CLPs and (or) spermidines on fungal infection *in vivo* and may suggest targets for future genetic manipulation of flax to obtain new lines resistant to diseases. However, the complexity of the ecosystems in the agricultural field also requires

several tests to determine the effects of a putative fungicide on plant resistance and yields, as well the effects on the environment, including the impact on the soil microbiota and beneficial interactions with the existing fungi (Stachowicz 2001).

Acknowledgements

Thank you to Dr. Khalid Rashid, Dr. Martin Reaney, and Dr. Louise Nelson, and their students, especially Melissa Larrabee, for supplying the fungal isolates. We would also like to thank Dr. Martin Reaney for supplying the CLP mixture used in this experiment. Thank you to Erica Packard for the great help setting up the experiments.

Funding

This work is supported by Brazilian agency Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support through the scholarship in the program Ciência sem Fronteiras, process number 99999.013148/2013-03, and by the Natural Sciences Research Council (NSERC) of Canada.

Author contributions

ILACRQ, JACRS, and MKD conceived and designed the study. ILACRQ performed the experiments/collected the data. ILACRQ and MKD analyzed and interpreted the data. MKD contributed resources. ILACRQ, JACRS, and MKD drafted or revised the manuscript.

Competing interests

The authors declare there are no competing interests.

Data availability

Data generated or analyzed during this study are provided in full within the published article.

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