Effect of linoleic acid on reproduction and yeast-mycelium dimorphism in the Dutch elm disease pathogens

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Effect of linoleic acid on reproduction and yeast-mycelium dimorphism in the Dutch elm disease pathogens

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Abstract

Elm populations from North America and Europe were devastated by Dutch elm disease (DED), a vascular disease caused by fungi from the genus *Ophiostoma* (Ascomycota). These pathogens feature a yeast-mycelium dimorphism that may be related to virulence by facilitating colonization of the host xylem. Cyclooxygenases (Cox) have been proposed to modulate yeast-mycelium dimorphism of DED fungi, and homologs of Cox-encoding genes have been found in the nuclear genome of *O. novo-ulmi* subsp. *novo-ulmi*. Linoleic acid, a substrate for Cox, was reported to stimulate the formation of asexual and sexual reproduction structures in DED strains grown on complex media. We hypothesized that linoleic acid also induced mycelium production in liquid shake culture conditions. Linoleic acid was found to enhance production of reproductive structures in sexual crosses conducted on complex medium (elm sapwood agar) but was not sufficient for these structures to form on minimal medium. In liquid shake cultures grown in minimal medium, addition of linoleic acid stimulated mycelial formation. Our results suggest that linoleic acid plays a role in two complex pathways, reproduction and dimorphism, in the DED pathogens.

Keywords : Fatty acid, *Ophiostoma*, yeast, hypha, perithecium, morphological transition

23 Résumé

24

25 Les populations d'ormes américains et européens ont été dévastées par la maladie
26 hollandaise de l'orme (MHO), une maladie vasculaire causée par des champignons
27 ophiostomatoïdes (Ascomycota). Ces agents pathogènes affichent un dimorphisme
28 levure-mycélium probablement lié à la virulence en facilitant la colonisation du xylème
29 de l'hôte. On a proposé antérieurement que des cyclooxygénases (Cox) modulent ce
30 dimorphisme. Des homologues de gènes *cox* sont présents dans le génome nucléaire
31 d'*O. novo-ulmi* subsp. *novo-ulmi*. L'acide linoléique, un substrat pour Cox, semble
32 stimuler la formation de structures de reproduction sexuée et asexuée chez les
33 ophiostomatoïdes sur milieux complexes. Nous avons émis l'hypothèse selon laquelle
34 l'acide linoléique induit la production de mycélium en milieux agités liquides. L'acide
35 linoléique a augmenté la production de structures de reproduction dans des
36 croisements sexués sur milieu complexe (milieu au bois d'orme), mais pas sur milieu
37 minimum. En milieu minimum liquide agité, l'acide linoléique a stimulé la formation de
38 mycélium. L'acide linoléique semble jouer un rôle dans les voies de reproduction et du
39 dimorphisme chez ces champignons.

40

41 *Mot-clés:* Acide gras, *Ophiostoma*, levure, hyphe, perithèce, transition morphologique

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Introduction

Successive pandemics of Dutch elm disease (DED) caused by *Ophiostoma ulmi* and *O. novo-ulmi* (Ascomycota) have inflicted massive losses to American and European elm populations (Santini et al. 2012). These pathogens exhibit yeast to mycelium dimorphism in which the yeast form is used for passive spread within xylem vessels of elm trees, whereas mycelium penetrates bordered pits and invades uninfected adjacent vessels. The mycelium form may also invade other cell types such as xylem parenchyma cells and fiber. Resistance of elms against DED appears to be, at least in part, related to the timing of defense mechanisms such as the production of tyloses and gels, as well as the synthesis and accumulation of phenolic compounds (Martin et al. 2007; Aoun et al. 2010). One consequence of rapid defense reactions may be the halting of yeast to hypha conversion, thus preventing invasive growth of DED fungi.

Several stimuli are known to control yeast to mycelium transition in shake liquid cultures of DED fungi. They include nitrogen source (Kulkarni and Nickerson 1981, Naruzawa and Bernier 2014), yeast cell concentration akin to quorum sensing (Kulkarni and Nickerson 1981, Hornby et al. 2004; Berrocal et al. 2012) and calcium-calmodulin interaction (Muthukumar and Nickerson 1984; Brunton and Gadd 1989; Gadd and Brunton 1992). According to Jensen et al. (1992), inhibitors of lipoxygenases (Lox) also interfere with yeast-mycelium transition and favor the production of yeast cells over mycelium. This was recently confirmed by Naruzawa and Bernier (2014) who further reported that salicylic acid, an inhibitor of cyclooxygenases (Cox), had a similar effect.

Lox and Cox are dioxygenase enzymes that catalyze the synthesis of oxylipins by incorporating oxygen atoms into polyunsaturated fatty acids (PUFAs) (Bugg 2003). According to results by Jensen et al. (1992) and Naruzawa and Bernier (2014), oxylipins could be related to the yeast-mycelium transition of DED pathogens.

Oxylipins function as hormone signals to modulate timing and balance between sexual and asexual development and toxin production in fungi. According to Tsitsigiannis et al. (2004a), an *Aspergillus nidulans* mutant in which the dioxygenase gene *ppoC* had been deleted exhibited increased sexual spore production and decreased asexual spore formation. The opposite response occurred in the case of an *A. nidulans* Δ *ppoA* mutant (Tsitsigiannis et al 2004b). An *A. nidulans* Δ *ppoA*, Δ *ppoB* and Δ *ppoC* triple mutant was unable to produce sterigmatocystin, a precursor of aflatoxin, both *in vivo* and *in planta*. On the other hand, this mutant overproduced penicillin (Tsitsigiannis and Keller 2006). Also, the impaired mutant could no longer colonize peanut seeds. The latter phenotype could be explained by decreased conidiation and lipase enzyme production (Tsitsigiannis and Keller 2006). In contrast, an *A. fumigatus* Δ *ppoA*, Δ *ppoB* and Δ *ppoC* triple mutant was hypervirulent in the invasive pulmonary aspergillosis murine model system and had tolerance to hydrogen peroxide, which implicates resistance to reactive oxygen species defense of host (Tsitsigiannis et al. 2005). Therefore, these enzymes and oxylipins are involved in the regulation of fungal metabolism. Interestingly, homologs of *ppo* genes are found in the *Ophiostoma novo-ulmi* genome (Comeau et al., 2015; Naruzawa et al. unpublished).

Linoleic acid was reported by Calvo et al. (1999) to regulate asexual and sexual spore production in *A. flavus*, *A. parasiticus* and *A. nidulans* in a way similar to oxylipins produced by Ppo dioxygenases. In the DED pathogens, addition of linoleic acid to a complex growth medium (potato dextrose agar) was found to induce the formation of asexual spore bearing structures called synnemata (formerly known as coremia) (Hubbes 1975). Furthermore, Hubbes et al. (1977) observed a high concentration of linoleic acid in elm sapwood and bark in June, and a decrease of this PUFA in heartwood. This would support the involvement of linoleic acid in the production of synnemata, since the latter are found in elm bark beetle breeding galleries under the bark (Hubbes et al. 1977). Linoleic acid also favored the production of sexual spore-bearing structures (perithecia) in complex media such as agar media containing hot water extracts of American elm wood, American elm leaves, green tea or black tea (Marshall et al. 1982).

Based on the above results, we speculated that linoleic acid was involved in the reproduction and yeast-mycelium dimorphism of DED pathogens. Our specific objectives were to verify whether linoleic acid stimulated the formation of asexual and sexual reproductive structures on defined solid growth media and enhanced the production of mycelium in liquid shake cultures.

Materials and Methods

Fungal strains

113

114 Strains used in this study were retrieved from the CEF (Centre d'Étude de la Forêt)
115 fungal collection at Université Laval, Québec, Canada
116 (<http://www.cef.ulaval.ca/index.php?n=CEF.CollectionsChampignonsPathogenes>).

117 These included *O. ulmi* Q412T (Mat1-1) and W9 (Mat1-2), *O. novo-ulmi* subsp. *novo-*
118 *ulmi* H327 (Mat1-1) and F5-28 (Mat1-2), and *O. novo-ulmi* subsp. *americana* VA3O
119 (Mat1-1) and NRRL6404 (Mat1-2). Strains were grown on an orbital shaker (110 rev
120 min⁻¹) at room temperature (24-25° C) in 125-mL Erlenmeyer flasks that contained 50
121 mL liquid *Ophiostoma* minimal medium (OMM; Bernier and Hubbes 1990) with 10 mM
122 addition of proline (L-proline; Sigma Aldrich, St. Louis, USA) to promote yeast cell
123 formation (Kulkarni and Nickerson 1981; Naruzawa and Bernier 2014).

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125 *Effect of linoleic acid on the formation of reproductive structures*

126

127 Production of reproductive structures was assessed by setting up sexual crosses on a
128 defined medium and a complex one. The former was solid OMM with ammonium
129 sulphate as nitrogen source, whereas the latter consisted in elm sapwood agar (ESA)
130 prepared according to Brasier (1981). Fungal strains were first cultured on MEA (Malt
131 extract agar, Oxoid, Nepean, Canada). In order to proceed with crosses, recipient
132 strains were grown on unamended solid OMM or ESA, or on solid OMM or ESA
133 supplemented with linoleic acid (Sigma Aldrich) at 6 µL mL⁻¹. Once mycelial colonies had
134 covered the entire plate (7-10 days), they were fertilized with 1-ml aliquots of yeast cell

suspensions of donor isolates grown in shake liquid OMM supplemented with proline. Three pairs of sexually compatible strains were used in reciprocal crosses, for a total of six combinations (Table 1). Each combination included 6 repetitions (a Petri dish was considered a repetition). Cultures were kept in an incubator at 23-24° C in the dark. Plates were examined for the presence of synnemata, protoperithecia and perithecia, starting at 8 days after fertilization until 60 days after fertilization. The experiment was conducted twice or three times.

Effect of linoleic acid on yeast-mycelium transition

The effect of linoleic acid on dimorphism was tested in liquid OMM supplemented with proline or arginine (L-arginine monohydrochloride, Sigma Aldrich) as nitrogen source. Linoleic acid or sterile distilled water (control) was added aseptically to the media at 6 μ L mL⁻¹ after autoclaving (121°C for 20 minutes). Six-day old shake cultures grown in OMM supplemented with proline (stationary phase) of strains W9, H327, VA3O or NRRL6404 were used to inoculate test media to a final concentration of 2x10⁷ spores mL⁻¹. Samples were incubated on an orbital shaker (110 rev min⁻¹) at room temperature (24-25° C). Yeast cells and mycelia were harvested by repeated sampling after 19 and 24 hours of incubation. For each incubation time, photographs were taken for each repetition with an Evolution VF digital color camera (Media Cybernetics, Rockville, USA) mounted on a Olympus BX41 (Tokyo, Japan) light microscope and analyzed with the software Q Capture Pro version 5.1.1.14 (Qimaging, Surrey, Canada). At least 100 fungal structures (yeast or hypha) were counted for each repetition. A spore with an

emergent hypha longer than the length of the spore diameter was counted as mycelium. Each treatment included five repetitions. The proportion of mycelia-yeast was calculated for each repetition. The experiment was conducted twice.

Statistical analysis of yeast-mycelium transition in liquid cultures

Treatments were arranged in a completely randomized design. Experiments on yeast-mycelium transition, which involved more than one sampling time, were treated as repeated measures. PROC GLM with « repeated » command was used on SAS software version 9.1 (SAS Institute, Cary, USA). Since the interaction with the time of sampling was significant, a separate *post-hoc* analysis (*t*-test) was conducted for each time and each strain for the control and the treatment.

Results

Effect of linoleic acid on the formation of reproductive structures

No reproductive structures (synnemata, protoperithecia or perithecia) were observed in sexual crosses conducted on unamended solid OMM nor on solid OMM supplemented with linoleic acid (Table 1). Unamended ESA medium induced a variety of responses in

the sexual crosses tested. Protoperithecia and perithecia were observed in the H327♀ (recipient) x F5-28♂ (donor) cross, as well as in the reciprocal F5-28♀ x H327♂ cross. In the former cross few perithecia were reported. Synnemata and protoperithecia were observed in the NRRL6404♀ x VA3O♂ cross, as well as in the reciprocal cross VA3O♀ x NRRL6404♂. However, very few reproductive structures (around five of each per petri dish) were observed in the latter. Synnemata, protoperithecia and perithecia were produced in crosses between strains Q412T and W9, irrespective of which strain was used as recipient or donor.

Solid ESA supplemented with linoleic acid induced the formation of reproductive structures in all crosses tested. On this medium, synnemata, protoperithecia and perithecia were formed in all crosses. Synnemata, protoperithecia and perithecia were observed in the NRRL6404♀ x VA3O♂ cross; however very few reproductive structures (five or less of each per petri dish) occurred in the VA3O♀ x NRRL6404♂ reciprocal cross, with protoperithecia and perithecia observed in only one of three separate experiments. Typical synnemata, perithecia and protoperithecia produced by *O. ulmi* and *O. novo-ulmi* on ESA media are shown in Figure 1.

Linoleic acid induces mycelium production in shake cultures of DED pathogens

Linoleic acid stimulated mycelium production in DED strains grown in shake liquid culture, including *O. ulmi* strain W9 which is strongly committed to yeast cell production even when exposed to mycelium-inducing conditions such as incubation in OMM in the presence of ammonium sulfate or arginine as nitrogen sources (Naruzawa and Bernier 2014). Increases in mycelium production in response to linoleic acid were statistically significant for all strains incubated for 24 hours in the presence of arginine (Fig. 2A) or proline (Fig. 2B) as nitrogen sources. However, observations made after 19 hours of incubation showed that addition of linoleic acid consistently increased mycelium production in all strains, except W9, in arginine-containing media, whereas it induced a significant response in all four strains tested in proline-containing media.

Discussion

Dutch elm disease fungi exhibit a yeast-mycelium dimorphism which may be related to virulence. They produce yeast cells in order to spread within xylem vessels of elms, while the mycelium morphology allows them to penetrate and invade uninfected adjacent vessels as well as xylem parenchyma cells and fiber. Delay in the transition from yeasts to hyphae could result in halting of the pathogen as timing for the production of tyloses and gels, and synthesis and accumulation of phenolic compounds appears to be essential for plant defense (Martin et al. 2007; Aoun et al. 2010). The transition from hyphae to yeasts also appears to be crucial for virulence. A spontaneous mutant of *O. novo-ulmi*, in which conversion from hyphae to yeasts was blocked, failed

to induce DED symptoms when inoculated to *Ulmus americana* (Richards 1994). Therefore, conversions from yeasts to hyphae and from hyphae to yeasts could both be implicated in virulence of the DED fungi.

Yeast-to-hyphae dimorphism seems to be controlled, at least in part, by dioxygenases since inhibitors of lipoxygenases and cyclooxygenases affect this transition in DED pathogens according to our previous study (Naruzawa and Bernier 2014). Dioxygenases oxidize polyunsaturated fatty acids (PUFAs) to produce oxylipins (Bugg 2003). The PUFA linoleic acid is a substrate for cyclooxygenases and a precursor of oxylipins. One can therefore speculate that linoleic acid is involved in mycelium production since inhibition of cyclooxygenases, and subsequently of oxylipins, disturbs yeast-to-hyphae transition in the DED pathogens.

Linoleic acid is known to induce asexual (synnemata) and sexual (perithecia) bearing structures in *Ophiostoma* species. Neumann and Hubbes (1972) observed that the sapstain fungus *O. piceae* produced the greatest number of synnemata in media supplemented with sapwood extracts from balsam fir (*Abies balsamea*). These authors established that linoleic acid was the compound responsible for the stimulation of asexual reproduction in this fungus. Hubbes (1975) later reported that, among many terpenes and unsaturated fatty acids he had tested, linoleic acid and its esters were the most active in stimulating the production of synnemata in the DED pathogens. Moreover, formation of perithecia in the DED pathogens was triggered by fatty acids in agar media with extracts of American elm wood, American elm leaves, and green and

black tea, according to Marshall et al. (1982). These authors also reported that abundant perithecia were obtained on a medium containing only the extract of black tea, linoleic acid, and pyridoxine.

In this study, we corroborated the effect of linoleic acid on synnemata and perithecia production in DED pathogens observed by Hubbes (1975) and Marshall et al. (1982) in complex media (OCM). Based on our observations, no reproductive structures occurred on synthetic medium without elm wood (OMM). Neumann and Hubbes (1972) had previously verified that *O. piceae* produced no synnemata on a poor medium (water agar) or on media containing heartwood extracts. It thus seems that a signal from the plant, specifically from the sapwood, is necessary for the formation of perithecia and synnemata in ophiostomatoid fungi. Hubbes et al. (1977) reported a high concentration of linoleic acid in elm sapwood and bark in June (the period when elms are most susceptible to DED in the northern hemisphere) and a decrease in the concentration of this product in heartwood. Linoleic acid was thus pointed out as the compound that favors formation of reproductive structures. Marshall et al. (1982) hypothesized that elm phenolics were also involved in perithecia formation. However, results from an earlier study by Holmes (1970) contradict this exclusivity of plant compounds in triggering perithecia formation. This researcher verified that perithecia of DED pathogens were formed in four out of eight variations of Zentmyer-Tchernoff synthetic media (glucose, asparagine, KH_2PO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, ZnSO_4 , FeCl_3 , pyridoxine, thiamine, and agar), although numbers were low and inconsistent, as pointed out by Marshall et al. (1982). On the other hand, we conducted crosses differently compared to Holmes (1970). We

seeded the recipient strain (♀ cultivated on solid media) with the donor strain (♂ from liquid OMM), whereas Holmes (1970) conducted crosses by placing a plug of culture of each fungal partner at 1 cm from each other (adjacent inoculation) on the Zentmyer-Tchernoff synthetic media. It is possible that some compounds which influenced production of sexual structures were present in the plug of parental agar cultures used by Holmes (1970) for crosses on synthetic media. This could explain the low and inconsistent production of these structures in the latter study.

All the crosses we examined on ESA amended with linoleic acid bore abundant perithecia except for the cross VA3O♀ x NRRL6404♂. These strains were expected to cross with each other since they both belong to *O. novo-ulmi* subsp. *americana* (Naruzawa and Bernier 2014) and originate from the USA. Holmes (1970) explained the lack of perithecia in some crosses of DED pathogens as a failure of gametangial contact which is required for the formation of ascocarps. Further incompatibility between VA3O♀ x NRRL6404♂ was observed in our study as none to few reproductive structures were seen on unamended ESA medium. However, in the reciprocal cross, NRRL6404♀ x VA3O♂, synnemata and protoperithecia were abundant on ESA. This points out a malfunction of sexual reproduction in cross VA3O♀ x NRRL6404♂. Possibly, VA3O could not act as a receptor for reproduction and NRRL6404 as a donor. In the study conducted by Holmes (1977), an isolate from the Netherlands acted only as male (donor) but kept its pathogenicity towards elm. The author argued that this strain had lost its hermaphroditic capacity after three months of laboratory

transfers. In our case, the cultures were grown for only one to two weeks on MEA after they had been recovered from storage at -80°C.

We observed a significant effect of linoleic acid on mycelium production in liquid cultures of DED pathogens. To the best of our knowledge, it is the first time this PUFA is associated with yeast to mycelium conversion in these fungi. Previously, a similar effect on yeast-mycelium dimorphism of DED strains was reported for yeast extract (Brunton and Gadd, 1989; Brunton and Gadd 1991; Naruzawa and Bernier 2014). The latter is a complex product which concentrates the water soluble portion of *Saccharomyces cerevisiae* cells that have been autolyzed. According to the manufacturer (BD Diagnostics, Sparks, MD, USA), yeast extract provides essential water soluble vitamins, amino acids, peptides and carbohydrates. Germ tubes of *O. novo-ulmi* induced by yeast extract exhibit increased uptake of Ca^{2+} compared to uptake by other cell types (Brunton and Gadd 1991). Yeast-mycelium transition with yeast extract was accompanied by increased intracellular concentration of cAMP in *O. novo-ulmi* (Brunton and Gadd 1989). According to these authors, addition of nucleotides and nucleosides caused a similar response but to a lesser extent compared to yeast extract. Inosine caused the largest response among the nucleotides tested. Thus, Brunton and Gadd (1989) could not explain whether the yeast extract effect on mycelium induction of *O. novo-ulmi* was due to the presence of nucleotides and nucleosides in this complex product.

Linoleic acid, on the other hand, is a simple molecule, a polyunsaturated fatty acid involved in prostaglandin synthesis. The results obtained here indicate that this fatty acid, and possibly products derived from it, is directly related to dimorphism. This, in turn, suggests that the pathways for the production of prostaglandins (eg. oxylipins) control, at least in part, the dimorphism of DED fungi. Arginine also induces mycelium production (Kulkarni and Nickerson 1981; Naruzawa and Bernier 2014) in DED fungi. After 19 h of incubation in liquid OMM supplemented with arginine, a strain (W9) did not respond to linoleic acid induction of mycelium. It could be that arginine masked the effect of linoleic acid on yeast-mycelium dimorphism in W9. After 24 h of incubation, the effect of linoleic acid on mycelium induction was clearer. Possibly, after this period, linoleic acid was consumed by cells grown in OMM supplemented with arginine and hyphae were induced. Arginine may disturb the linoleic acid effect and interact with this PUFA in an unknown way in young (less than 24-hour old) cultures of W9.

Lipids have been associated with yeast to hyphae dimorphism in other fungal species. According to Ito et al. (1982), an increase in the lipid synthesis rate was accompanied by yeast to hypha transition when *Mucor racemosus* was transferred from anaerobic to aerobic conditions. This transition was not detected when cerulenin, an antibiotic that inhibits fatty acid synthase and then lipid synthesis (Sanadi et al. 1987), was added to the media under aerobic conditions. This suggests that lipid synthesis is correlated to *M. racemosus* dimorphism. Nevertheless, cerulenin inhibited also ornithine decarboxylase activity, RNA and protein synthesis (Ito et al. 1982).

Another indication that lipids are connected to dimorphism was provided by the study of Zinjarde et al. (2008). Optimal (60%) formation of mycelia in *Yarrowia lipolytica* was obtained with coconut oil or palm kernel oil, whereas suboptimal (40%) mycelium formation was induced with peanut oil, olive oil, or sesame oil (Zinjarde et al. 2008). Major fatty acids present in coconut and palm kernel oil are lauric acid (45-48%) and myristic acid (18%) (Mlícková et al. 2004). While myristate was not effective for dimorphic transition (32%), the authors obtained 51% of the cells in mycelial form with laurate. In addition, they observed that a combination of laurate and myristate in a ratio of 2.6:1 enhanced mycelium formation to 65%. Longer chain saturated fatty acids (potassium palmitate or stearate) or oleic acid stimulated the yeast form of *Y. lipolytica*.

Farnesol, an oxylipin, plays a crucial role in the density-dependent dimorphism in *Candida albicans* (Nickerson et al. 2012). This phenomenon is also known as quorum sensing. According to Hornby et al. (2001), the quorum sensing molecule farnesol halts the mycelial phase at 10^6 cells ml^{-1} in liquid culture. In addition, a metabolite derived from linoleic acid by β -oxydation, 3(R)-hydroxytetradecanoic acid, participates in the quorum sensing mechanism increasing germ tube and biofilm formation (Nigam et al. 2011). This metabolite upregulates the *CAP1* gene, which encodes an adenylate cyclase-associated protein involved in the formation of Cap protein as well as hyphal wall protein 1 (Hwp1), involved in the regulation of filamentous growth (Nigam et al. 2011).

Ophiostoma novo-ulmi also features a yeast-mycelium dimorphism controlled by quorum sensing (Hornby et al. 2004). Isoleucine 2-methyl-1-butanol, a fusel alcohol, was demonstrated by Berrocal et al. (2012) to be responsible for the density dependent dimorphism behaviour of this DED pathogen. In concentrations higher than 2×10^7 spores mL^{-1} this molecule seems to be responsible for the yeast morphology that is the predominant growth form within these concentrations (Hornby et al. 2004). A correlation between *S. cerevisiae* cells enriched with linoleic and linolenic acid and wine fermented samples richer in fusel alcohols was observed by Rosi and Bertuccioli (1992). In contrast, Thurston et al. (1981) observed a reduction in the production of esters (60-75%) and fusel alcohols (69-84%) by the yeast in beer when linoleic acid was added. Thus, it is possible that the density dependent phenomenon in *O. novo-ulmi* is connected to fatty acid uptake by the fungus.

A link between sexual differentiation signaled by mating pheromones and regulation of dimorphism seems to take place in fungi since both features share signalling components. For the corn smut pathogen, *Ustilago maydis*, these characteristics are also connected to plant invasion (Klosterman et al. 2007; Brefort et al. 2009). Mating and dimorphism are linked by mitogen activated protein (MAP) kinase cascades as verified for *U. maydis* and *S. cerevisiae* (Madhani and Fink 1998; Klosterman et al. 2007; Brefort et al. 2009). There is an interplay between the MAP kinase pathway and the cyclic adenosine monophosphate (cAMP) pathway to regulate the dimorphism and mating in these fungi. The filamentous growth of diploid cells, invasive growth of haploid cells in culture media and mating pheromone response are controlled by the same four

377 MAP kinase pathway components (protein kinases Ste20p, Ste11p and Ste7p, and
378 transcription factor Ste12p) in *S. cerevisiae* (Madhani and Fink 1998). As well, MAP
379 kinase cascade components such as Kpp4/Ubc4, Fuz7/Ubc5, and Kpp2/Ubc3 are
380 shared in *U. maydis* for mating, conversion from yeast-to-hypha and invasion of the
381 plant (Sánchez-Martínez and Pérez-Martín 2001; Brefort et al. 2009).

382
383 A homolog to cyclooxygenases in *Magnaporthe oryzae*, 10*R*-Dox-Eas, was recently
384 discovered by Hoffmann et al. (2014). The biological function of this cyclooxygenase-
385 like protein is unknown in *M. oryzae*. On the other hand, Soanes et al. (2012) verified
386 that the gene that encodes this protein is up-regulated during the early and late phases
387 of appressorium formation and down-regulated by gene deletion of the MAP kinase
388 *PMK1*. Moreover, homologs of this *M. oryzae* protein regulated by a MAP kinase are
389 found in plant pathogenic fungi, which indicates a possible link between this protein and
390 pathogenicity (Hoffmann et al. 2014). Interestingly, we found the presence of a homolog
391 of 10*R*-Dox-Eas and PpoA in the DED agents (Naruzawa et al. unpublished).

392
393 Mating and dimorphism may thus also be interconnected in the DED pathogens.
394 Linoleic acid, other fatty acids and their products would regulate these features. These
395 results reinforce the relevant link between reproduction, dimorphism control and
396 virulence in DED fungi. Research needs to be directed to the above fitness
397 characteristics in order to design efficient DED control approaches. One such approach
398 could target fatty acid regulation or their inhibition to prevent reproduction, dimorphism

and, subsequently, higher virulence in these fungi. As the genome of the aggressive *O. novo-ulmi* subsp. *novo-ulmi* strain H327 was recently sequenced (Forgetta et al. 2013) and annotated (Comeau et al. 2015), studies of genes implicated in dimorphism, virulence, reproduction and other fitness features are facilitated. Furthermore, these results could be used as a model for studies of other ophiostomatoid fungi and pathogenic Ascomycetes.

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591 Table 1. Production of reproductive structures in crosses of *Ophiostoma novo-ulmi*
 592 subsp. *novo-ulmi*, *O.novo-ulmi* subsp. *americana* and *O. ulmi* on four solid media*.
 593 Abbreviations: Protoperithecia = PP, Perithecia = P, Synnemata = S.

Crosses	OMM†	OMM + linoleic acid	ESA	ESA + linoleic acid
H327♀ x F5-28♂‡	-	-	PP, P ⁺⁺ §	S, PP, P ⁺⁺⁺
F5-28♀ x H327♂	-	-	PP, P ⁺⁺⁺	S, PP, P ⁺⁺⁺
NRRL6404♀ x VA3O♂	-	-	S, PP ⁺	S, PP, P ⁺
VA3O♀ x NRRL6404♂	-	-	S, PP ⁺	S, PP, P ⁺
W9♀ x Q412T♂	-	-	S, PP, P ⁺⁺⁺	S, PP, P ⁺⁺⁺
Q412T ♀ x W9♂	-	-	S, PP, P ⁺⁺⁺	S, PP, P ⁺⁺⁺

594 *Observations presented in this table were made 60 days after recipient cultures had
 595 been fertilized with donor strains

596 †OMM= *Ophiostoma* minimal medium (OMM; Bernier and Hubbes 1990); ESA= elm
 597 sapwood agar (Brasier 1981). Linoleic acid was added to a final concentration of 6 µl
 598 mL⁻¹.

‡Strains followed by ♀ and ♂ are recipients and donors, respectively.

§Semi-quantitative data are shown for perithecia. + = a few; ++= moderate quantity;
+++=many perithecia was observed.

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Figure captions :

Figure 1. Production of reproductive structures in crosses between Dutch elm disease fungi on solid Elm Sapwood Agar (ESA) media. A. Cross W9♀ x Q412T♂ yielded synnemata, protoperithecia and perithecia on ESA supplemented with linoleic acid. B. Protoperithecia and perithecia were obtained in cross H327♀ x F5-28♂ on solid ESA supplemented with linoleic acid. C. Synnemata produced by cross VA3O♀ x NRRL6404♂ on ESA with linoleic acid. D. Only protoperithecia were formed in cross H327♀ x F5-28♂ on ESA without linoleic acid. Large arrows indicate synnemata, medium arrows perithecia, and small arrows protoperithecia.

Figure 2. Effect of linoleic acid on mycelium production in Dutch elm disease pathogens in shake liquid *Ophiostoma* minimal medium (OMM) supplemented with arginine (A) or proline (B). Strains were grown for 19h (white and black bars) and 24h (light gray and dark gray bars). Treatments without linoleic acid (LA) are depicted as white and light gray bars and with LA (6 µl mL⁻¹) as black and dark gray bars. Values are means of five replicates from a representative experiment. *P* values are shown for comparison between treatments for each combination of strain-period (19h or 24h) according to *t*-test. (C) Yeast cells of *O. novo-ulmi* NRRL6404 after 24h of incubation in OMM with proline (upper panel) and mycelium production of the same strain after 24h in OMM with proline and linoleic acid (lower panel). Scale bars = 20µm.

A **C** **B**