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Diversity in yeast-mycelium dimorphism response of the Dutch elm disease pathogens: the inoculum size effect

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Abstract

Dutch elm disease (DED) is caused by the dimorphic fungi *Ophiostoma ulmi*, *O. novo-ulmi* and *O. himal-ulmi*. A cell population density-dependent phenomenon related to quorum sensing was previously shown to affect the reversible transition from yeast-like- to mycelial growth in liquid shake cultures of *O. novo-ulmi* strain NRRL 6404. Since the response to external stimuli often varies among DED fungal strains, we evaluated the effect of inoculum size on eight strains of the three species of DED agents by determining the proportion of yeast and mycelium produced at different spore inoculum concentrations in defined liquid shake medium. Results show that not all DED fungi strains respond similarly to inoculum size effect since variations were observed among strains. It is thus possible that the different strains belonging to phylogenetically close species use different signalling molecules or molecular signalling pathways to regulate their growth mode via quorum sensing mechanisms.

Résumé

La maladie hollandaise de l'orme (MHO) est causée par les champignons dimorphiques *Ophiostoma ulmi*, *O. novo-ulmi* et *O. himal-ulmi*. Il a déjà été démontré qu'un phénomène dépendant de la densité de la population cellulaire de l'inoculum, lié à l'effet du quorum, affecte la transition réversible de la croissance levuriforme à la croissance mycélienne chez des cultures liquides agitées de la souche *O. novo-ulmi* NRRL6404. Puisque la réponse aux stimuli externes varie fréquemment parmi les souches de champignons de la MHO, nous avons testé l'effet de la densité de l'inoculum chez huit souches des trois espèces d'agents de la MHO en déterminant la proportion de levure et de mycélium produits à différentes concentrations de spores de départ dans un milieu liquide défini agité. Les résultats démontrent que les souches de champignons de la MHO ne répondent pas de manière similaire à l'effet de la taille de l'inoculum puisqu'il existe

des variations entre les différentes souches testées. Il est ainsi possible que les différentes souches appartenant à des espèces phylogénétiquement proches utilisent des molécules de signalisation et des cascades de signalisation moléculaires différentes afin de réguler leur type de croissance par l'effet du quorum.

Keywords

Density-dependent phenomenon, quorum sensing, yeast, mycelium, *Ophiostoma spp.*

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Dutch elm disease (DED) is responsible for the drastic decline of populations of elm species native to Europe and North America during the last century (Brasier 2000). This vascular disease is caused by the dimorphic fungus *Ophiostoma ulmi* and its more virulent sister species, *O. novo-ulmi* (Brasier 1991). A third species, *O. himal-ulmi*, is an endophyte of *Ulmus wallichiana* in Asia but was shown to be pathogenic to European elm species and varieties (Brasier and Mehrotra 1995). Since dimorphism is believed to be required for pathogenicity of the DED fungi (Nadal et al. 2008; Richards 1994), it is important to study this trait to better understand the disease and eventually control it. It was previously demonstrated that dimorphism in liquid shake cultures of *O. novo-ulmi* strain NRRL 6404 (formerly referred to as an aggressive strain of *Ceratocystis ulmi*) can be controlled by different factors, including calcium, nitrogen source and inoculum size (Kulkarni and Nickerson 1981; Hornby et al. 2004; Gadd and Brunton 1992). Our group recently showed that representative strains of the three known species of DED fungi responded differently to nitrogen source and to propyl gallate. Moreover, a concentration of 20 mM calcium in the culture medium had a significant effect on dimorphism in *O. novo-ulmi* strain NRRL 6404 but not in the other strains investigated (Naruzawa and Bernier 2014). The contrasted response of DED fungi to the subset of stimuli tested by Naruzawa and Bernier (2014) suggests that this behavior might also characterize the response to other external factors.

In this study, we examined the effect of inoculum size on dimorphism in *Ophiostoma* spp., a phenomenon related to quorum sensing. Described originally in bacteria (Fuqua et al. 1994) but now considered widespread in microorganisms, quorum sensing is a process used by individuals to communicate among themselves and evaluate population density (Hogan 2006; Barriuso 2015). This communication results in coordinated gene expression and is modulated by signalling molecules secreted by the microorganisms. For example, in the dimorphic human fungal

pathogen *Candida albicans*, one of the molecules involved in quorum sensing is farnesol, an oxylipin responsible for suppressing the expression of genes involved in mycelial development and upregulating the expression of mycelial growth inhibitor genes (Hornby et al. 2001; Han et al. 2011). Quorum sensing has previously been studied in DED fungi, but only in *O. novo-ulmi* strain NRRL 6404 (Kulkarni and Nickerson 1981; Hornby et al. 2004; Berrocal et al. 2012). Inoculum size was shown to have an effect on dimorphism in strain NRRL 6404, as it was in part responsible for the reversible shift from yeast-like- to mycelial growth. When incubated in liquid media containing proline as the sole nitrogen source, strain NRRL 6404 tended to adopt yeast-like growth when the concentration of the initial inoculum was superior to 10^6 spores mL⁻¹, whereas below this concentration mycelial growth was favored (Kulkarni and Nickerson 1981). Recently, the molecule involved in quorum sensing signalling in *O. novo-ulmi* NRRL 6404 was determined to be 2-methyl-1-butanol, a fusel alcohol derived from isoleucine (Berrocal et al. 2012). Farnesol had no effect on quorum sensing in *O. novo-ulmi* NRRL 6404 (Berrocal et al. 2012; Hornby et al. 2004). However, farnesol was recently reported to mediate quorum sensing in strain CECT 20416 of *O. piceae*, a sapstaining species that is phylogenetically very close to the DED fungi (de Salas et al. 2015). In yet another closely related species, *O. floccosum*, the most abundant quorum sensing molecule detected in strain F1F1A55-0 was the cyclic sesquiterpene 1,1,4a-trimethyl-5,6-dimethylene-decalin (Berrocal et al. 2014). These facts, along with results obtained by Naruzawa and Bernier (2014), led us to hypothesize that the mechanisms and molecules regulating quorum sensing in DED fungi might not be the same for each strain. Therefore, the objective of our study was to verify the effect of inoculum size on a greater variety of strains to observe if the effect is comparable in all DED fungal strains tested.

The eight strains tested in this experiment were retrieved from the CEF (Centre d'Étude de la

Forêt) fungal collection kept at Université Laval (Québec, Qc, Canada; <http://www.cef.ulaval.ca/index.php?n=CEF.CollectionsChampignonsPathogenes>). These included strains of *O. ulmi* (Q412T and W9), *O. novo-ulmi* subsp. *novo-ulmi* (H327 and AST20), *O. novo-ulmi* subsp. *americana* (NRRL 6404 and FG245) and *O. himal-ulmi* (HP25 and HP50) which were part of a previous study by Naruzawa and Bernier (2014). Strains were grown in 125-mL Erlenmeyer flasks containing 50 mL of *Ophiostoma* minimal medium (OMM) (Bernier and Hubbes 1990), to which 1.15 g L⁻¹ of proline (Sigma Aldrich, St. Louis, MO, USA) was added as the sole nitrogen source to promote yeast-like growth. The liquid cultures were incubated at room temperature (22 °C) on an orbital shaker (150 rev min⁻¹) in an Ecotron incubator (Infors HT, Basel, Switzerland). Once cultures had reached the stationary phase (after 6 days of incubation), aliquots were aseptically distributed in 125-mL flasks containing 50 mL of OMM medium with proline to obtain final concentrations of 10⁵, 10⁶, 10⁷ and 10⁸ spores mL⁻¹ for each strain. Flasks were incubated at room temperature for 18 hours on an orbital shaker (110 rev min⁻¹). Aliquots were distributed in Costar 48-well plates (Corning, Corning, NY, USA). Samples from treatments inoculated with 10⁷ spores mL⁻¹ and 10⁸ spores mL⁻¹ were respectively diluted four and six times for a total volume of 500 µL per well for each treatment. Samples were analyzed by phase contrast microscopy at 400X (ApoTome, Zeiss, Toronto, ON, Canada), and photographs were taken (AxioVision, Zeiss) and used for counting yeasts and mycelia. For each repetition, 100 fungal structures were counted when possible. Spores with emergent hyphae longer than the length of the spore diameter were counted as mycelia. The proportion of yeasts was computed for each repetition. Differences among treatments were statistically analyzed using ANOVA and the post hoc Tukey's range test ($p < 0.05$) (SAS version 9.1, SAS Institute Inc, Cary, NC, USA). Arcsin transformations were applied to variates that did not meet the requirements for ANOVA to normalize the residuals. For each strain, five biological replicates of each treatment were

tested. Each experiment was conducted at least twice.

In general, the proportion of yeasts increased with an increase in the initial concentration of spores. Three general trends were observed among the eight DED strains studied (Fig 1). Strains FG245, NRRL 6404, H327, HP25 and HP50 showed a gradual response to an increase in the initial concentration of spores. A higher initial concentration of spores enhanced yeast production, as observed in phase contrast microscopy (Fig 2). Typically in cultures of these strains, the proportion of yeasts went from 25-65% when inoculated at 10^5 spores mL^{-1} up to 80-100 % when inoculated at 10^8 spores mL^{-1} . For *O. ulmi* strains Q412T and W9, the effect was much less pronounced. The proportion of yeast cells in cultures of strain Q412T never exceeded 70%, even with initial inoculum at 10^8 spores mL^{-1} . In contrast, strain W9 appeared to be committed to produce yeasts, as reported previously by Naruzawa and Bernier (2014) for other external stimuli such as nitrogen source and dioxygenase inhibitors. In this strain, yeast cells accounted for at least 75% of the structures formed in cultures inoculated with the lowest (10^5 spores mL^{-1}) concentration of spores. On the contrary, *O. novo-ulmi* subsp. *novo-ulmi* strain AST20 was strongly committed to produce mycelia but did respond markedly to the highest (10^8 spores mL^{-1}) concentration of spores. Therefore, dimorphism in DED fungi seems to be influenced by inoculum size. Variations in the proportion of yeast cells due to inoculum size were observed in every strain tested, even though the intensity of the effect varied from one strain to another. We found no evidence that strain behaviour was related to taxonomic position. For example, the two *O. novo-ulmi* subsp. *novo-ulmi* strains (H327 and AST20) responded differently to inoculum size. Therefore, our results on the response of DED fungi to inoculum size confirm previous observations by Naruzawa and Bernier (2014) that the response of these organisms to external stimuli cannot be predicted from their taxonomic identity. Moreover, the response of

strains to a given factor cannot be predicted from their response to other external stimuli. For example, strain AST20 exhibited a unique response to inoculum size among the strains tested, whereas it did not stand out in the experiments reported by Naruzawa and Bernier (2014).

Inoculum size effect had previously been studied in *O. novo-ulmi* subsp. *americana* strain NRRL 6404 (Kulkarni and Nickerson 1981; Hornby et al. 2004; Berrocal et al. 2012). Although we have now further characterized the phenotypic response of DED pathogens to inoculum size by investigating a higher number and a wider selection of strains, molecular mechanistic and metabolic pathways controlling these systems remain to be verified. Since our results show intraspecific and interspecific variations in the inoculum size effect, like Naruzawa and Bernier (2014) demonstrated for other factors, we believe that different DED fungal strains may have specific responses to stimuli and that not everything reported for *O. novo-ulmi* NRRL 6404 can be transposed to other DED fungal strains. It would therefore be important to identify the nature of the molecules and molecular pathways involved in quorum sensing in several strains in order to determine whether or not a single quorum sensing mechanism occurs in DED pathogens.

In addition to the molecules already mentioned, various other compounds are known to be involved in quorum sensing in fungi. These include a wide array of fusel alcohols (Berrocal et al. 2012), lactones, fatty alcohols, and phenyltanoids. The effect of lactones, namely γ -heptalactone and butyrolactone I, on morphology has been documented in *Aspergillus* species: the former in *A. nidulas* (Williams et al. 2012) and the latter in *A. terreus* (Raina et al. 2012). In *A. terreus*, the addition of butyrolactone I to cultures results in a higher yield of butyrolactone I itself and of lovastatin, a well characterized quorum sensing molecule in this species (Raina et al. 2012). In *C.*

albicans, a fatty alcohol (dodecanol) and a phenyltanoid (tyrosol) are also involved in quorum sensing besides farnesol. Dodecanol and farnesol promote yeast-like growth, whereas tyrosol inhibits it and therefore promotes mycelial growth (Davis-Hanna et al. 2008; Chen et al. 2004).

Furthermore, it is believed that oxylipins and certain enzymes that contribute to oxylipin production, such as cyclooxygenases (cox) and lipoxygenases (lox), may also be involved in dimorphism in DED fungi (Jensen et al. 1992; Naruzawa and Bernier 2014; Naruzawa et al. 2016). Oxylipins and their role in quorum sensing have been studied more in depth in *Aspergilli*. For example, quorum sensing in *A. flavus* is regulated by lox activity (Horowitz Brown et al. 2008) as well as Ppo cyclooxygenases and oxylipins produced by these cyclooxygenases (Horowitz Brown et al. 2009). Also, in *A. nidulans*, oxylipins are believed to activate protein kinase A (PKA) signalling pathways when coupled to GprD, an *A. nidulans* GPCR (G-protein-coupled receptor), as they are responsible for the accumulation of cyclic adenosine monophosphate (cAMP) in wild-type strains, whereas no cAMP accumulation is observed in $\Delta gprD$ mutants (Affeldt et al. 2012). Therefore, it would be of interest to study more in depth the implication of oxylipins and the enzymes that contribute to their production in order to better understand quorum sensing in different strains of DED fungi. To this end, our research group is currently working on producing and characterizing Δppo mutants of *O. novo-ulmi* H327 in order to assess if Ppo enzymes play a role in dimorphism and quorum sensing in DED fungi.

In conclusion, we demonstrated that the effect of inoculum size is not homogenous in DED fungi. In fact, intraspecific and interspecific variations in the effect of quorum sensing were observed among the eight strains of the three known species of DED fungi studied. Therefore, we believe that different quorum sensing molecules or molecular pathways might be involved in the

different strains. Identifying those molecules and molecular pathways is important in order to better understand DED with the purpose of developing new strategies for controlling the disease.

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Figure captions

Fig. 1. Effect of inoculum size on dimorphism in *Ophiostoma ulmi*, *O. novo-ulmi* and *O. himal-ulmi*. Strains were grown for 18 h in *Ophiostoma* minimal medium supplemented with proline as the sole nitrogen source. Values are means of five replicates from a representative experiment. The different letters associated to each mean represents means that differ significantly at $P < 0.05$ according to Tukey's HSD test. Standard errors are shown only when they exceed the size of the symbol.

Fig. 2. Effect of inoculum size on cell morphology in *Ophiostoma novo-ulmi* strain H327 grown for 18 h in *Ophiostoma* minimal medium supplemented with proline. Strains were incubated at four different concentrations: 10^5 spores mL^{-1} (**A**), 10^6 spores mL^{-1} (**B**), 10^7 spores mL^{-1} (**C**), 10^8 spores mL^{-1} (**D**). Aliquots of 500 μL were then distributed in Costar 48-well plates and analyzed by phase contrast microscopy at 400X. Samples from treatments inoculated with 10^7 spores mL^{-1} and 10^8 spores mL^{-1} were respectively diluted four and six times before they were analyzed. Photographs of a representative experiment; Scale bars = 100 μm . Inoculum was entirely composed of yeasts.

O. ulmi

O.novo- ulmi subsp americana

O. novo-ulmi subsp novo-ulmi

O. himal-ulmi