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Effect of growth conditions on *Ophiostoma piceae* (Münch) H. & P. Syd. and *Ophiostoma floccosum* Math-Käärik albino strains culture morphology

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Abstract

Blue stain in *Pinus radiata* D. Don wood is predominantly caused by *Ophiostoma* genus fungi. Stained wood causes significant economic losses to forestry and timber industries. Currently, different chemical fungicides are used to prevent the occurrence of blue stain fungi. However, recent reports have questioned the environmental friendliness of these fungicides. For this reason, the use of biological control, in particular with albino strains of the Ophiostoma genus, appears to be an environmentally safe and a technically feasible alternative to work with. The potential field application of this technology would use a formulation containing the albino fungus-the bioactive ingredient- in its yeast like form. However, some Ophiostoma strains cultures present a marked filamentous morphology in liquid culture medium, affecting the efficiency in the production

Resumen

La mancha azul en madera de *Pinus radiata* D. Don, causada predominantemente por hongos del género *Ophiostoma*, provoca pérdidas económicas significativas a las industrias forestales y madereras. Actualmente, diferentes fungicidas han sido utilizados para prevenir la aparición de la mancha azul. Sin embargo, reportes recientes han cuestionado que estos productos no son amigables con el ambiente. Por esta razón el uso del control biológico, específicamente con cepas albinas del género *Ophiostoma*, parece ser una alternativa ambientalmente segura y técnicamente viable de aplicar. El potencial campo de aplicación de esta tecnología se basa en el uso de una formulación que contiene el hongo albino-ingrediente bioactivo- en su morfología levaduriforme. Sin embargo, algunos cultivos de cepas del género *Ophiostoma* presentan

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of the bioactive ingredient. The hypothesis of this work presumed the possibility to control the morphology of *Ophiostoma* cultures, through the modification of growth conditions in liquid medium. The effect of inoculum size, growth temperature and agitation rate on the morphology of PcF2A29 *Ophiostoma piceae* and FIF1A55 *Ophiostoma floccosum* albino strains in liquid culture medium, was studied. A 2^3 factorial design was employed. The results showed that the initial inoculum size had a statistically significant effect on yeast-like growth morphology in both strains, whereas the temperature only produced a significant effect in PcF2A29 *O. piceae* strain.

Key words: *Ophiostoma piceae*, *Ophiostoma floccosum*, wood-staining fungi, fungal dimorphism, inoculum size, Chile.

una marcada morfología filamentosa en medio de cultivo líquido, afectando la eficiencia del sistema de producción del ingrediente bioactivo. La hipótesis de este trabajo presumió la posibilidad de controlar la morfología de cultivos de *Ophiostoma*, a través de la modificación de las condiciones de cultivo en medio líquido. Se estudió el efecto del tamaño del inóculo, temperatura de crecimiento y velocidad de agitación en la morfología de las cepas albinas PcF2A29 *Ophiostoma piceae* y FIF1A55 *Ophiostoma floccosum* en medio de cultivo líquido, Se empleó un diseño factorial 2^3 Los resultados mostraron que el tamaño del inóculo tuvo un efecto estadísticamente significativo en la morfología levaduriforme de ambas cepas, mientras que la temperatura sólo genera un efecto significativo en la cepa PcF2A29 *O. piceae*.

Palabras clave: *Ophiostoma piceae*, *Ophiostoma floccosum*, hongos manchadores de la madera, dimorfismo fúngico, tamaño del inóculo, Chile.

Introduction

Blue stain in radiata pine wood is mainly caused by *Ophiostoma* and *Sphaeropsis* genus fungi (Swart et al. 1985, Navarrete et al. 2008). These fungi penetrate the wood through lumens and pits and as they grow, they consume simple sugars, fatty acids, sterols and triglycerides (Jacobs and Wingfield, 2001). The staining fungi synthesize melanin (Zimmerman et al. 1993; Zink and Fengel, 1988), the pigment which imparts the characteristic color of the colonized wood (Scheffer, 1973).

Although the staining does not affect the mechanical properties of the wood, it reduces the quality of the products, affecting the forestry industry profitability (Seifert, 1993). In addition, the use of stained wood in the fabrication of wood pulp requires an increase in the consumption of reagents during the whitening process (Behrendt and Blanchette, 1997).

In Chile, several chemical products as carbendazim, chlorothalonil and copper 8-quinolate or mixtures of them are used to prevent the staining of wood (Montes et al. 2001, Lanfranco et al. 2004). However, these products that were initially considered environmentally safe are now questioned (APVMA, 2007; M^{c}Mahon et al. 2011).

Likewise, various studies have demonstrated that biological control of blue stain (Lee and Oh, 2000), in particular using albino fungus species from *Ophiostoma*, is environmentally friendly and technically feasible to apply (Held, et al. 2003, Navarrete et al. 2008).

The preparation of the bioprotective formulation requires the availability of albino fungus (biologically active ingredient) in its unicellular form. However, some species of Ophiostoma genus have a dimorphic (yeast-mycelium) growth, affecting the efficiency of production of spores (yeasts). Fungal dimorphism is defined as reversible change from a unicellular growth to a multicellular one in response to environmental changes and growth conditions (Deacon, 2006, Nadal et al. 2008).

In the present work, the effect of variables: inoculum size, growth temperature and agitation rate on the morphology of cultures of albino strains PcF2A29 *Ophiostoma piceae* (Münch) H. & P. Syd. and FIF1A55 *Ophiostoma floccosum* Math-Käärik, was examined.

Material and methods

Microorganisms. Albino strains PcF2A29 *O. piceae* and FIF1A55 *O. floccosum* were obtained from the albino strain culture collection at the Laboratory of Wood Biodegradation in the Departamento de Ingeniería en Maderas de la Universidad del Bio-Bio (Chile).

Liquid culture medium. Culture medium was prepared with 15 g of malt extract (Merck), 4 g of yeast extract (Merck), 0,25 g of chloramphenicol (Sigma) and 0,25 g streptomycin sulfate (Sigma). All media reagents, with the exception of streptomycin sulfate, were dissolved in a liter of distilled water, poured into a culture bottle and autoclaved at 121 °C for 25 minutes. The streptomycin sulfate was added to the warm media aseptically.

Preparation of the initial inoculum. Erlenmeyer flasks (500 ml), containing 125 ml of liquid culture media were inoculated with stock culture cells and them were

incubated in an orbital agitator at 120 rpm for 5 days and 25 °C. The spores were separated from the mycelium by filtration through a Whatman Nº 4 filter paper, they were recovered by centrifugation and washed three times with equal volumes of sterile distilled water. Finally, the spores were resuspended in 50 mM phosphate buffer solution (pH 6,5) and stored at 4 °C until required for experimentation.

Culture conditions and growth evaluation. Erlenmeyer flasks (250 ml), 16 in total containing 50 ml of liquid culture medium, were inoculated with concentrations of the strains to be studied and placed in two orbital agitators inside of 2 low temperature incubators and adjusted as indicated in Table 1. Culture samples were taken at 24, 48 and 72 hours. Evaluation of culture morphology was determined by microscopic examination at 400X (Nikon Eclipse E600 microscope) on a hemocytometer.

Experimental design. A 2^3 factorial design of two levels, non-replicated and completely random, for each albino strain, was used. The levels of factors studied were: **A-**Inoculum size ($5{,}0x10^5$ and $1{,}0x10^7$ C.F.U. ml^{-1}), **B-**Growth temperature (18 and 26 °C), and **C-**Agitation rate (100 and 200 rpm). Table 1 shows the random order and treatment conditions for each run. The variable responses evaluated were: yeast/mycelium ratio (%) and cell concentration (C.F.U. ml^{-1}) for 24, 48 and 72 hours of growth.

Results

Analysis of variance (ANOVA) demonstrated to both *Ophiostoma* species a high significance level between inoculum size factor and the response variables: yeast/mycelium ratio (%) and concentration of spores (C.F.U. ml^{-1}) after 24, 48 and 72 hours of fermentation. For FIF1A55 *O. floccosum* Table 2 shows the contribution (%) that the analyzed effects and their interactions had on the observed variability of responses.

Initial inoculum size was the factor that mostly contributed to the yeast/mycelium ratio and spore concentration in both species at the three evaluated times. The contribution was higher than 92% in all the cases, which demonstrates the relative importance of this factor. At 24 hours of growth, the agitation rate and the interaction inoculum size-temperature showed contribution percentages of 4.6 and 2.3%, respectively. However, their impact was not statistically significant. The R^2 and adjusted R^2 values obtained were higher than 0.90 and the predicted R^2 was in agreement with the adjusted R^2, demonstrating the validity of the generated models (Table 2).

In the case of the strain PcF2A29 of the *O. piceae* species, the relative importance and percentages of contribution of the different factors analyzed on response variables are shown in Table 3.

For PcF2A29 albino strain, the inoculum size effect was greater than 80% for both variable responses in all fermentation times evaluated, except for the yeast/mycelium relationship at 24 hours of growth, where the relative importance of temperature (34%) and the interaction between inoculum size-temperature (24%), together with inoculums size effect (40%) were statistically significant. The R^2 and adjusted R^2 values obtained were higher than 0.80 and the predicted R^2 was in agreement with the adjusted R^2 for the variable responses, demonstrating the validity of the generated models (Table 3).

Table 4 shows the values for the relationship for yeast/mycelium and spore concentration obtained with different treatments at three fermentation times for FIF1A55 albino strain.

Table 1. Treatment run order for FIF1A55 *O. floccosum* and PcF2A29 *O. piceae* albino strains growth morphology experiment.

Cuadro 1. Orden de corrida de los tratamientos para el ensayo de morfología de crecimiento de cultivos de las cepas albinas FIF1A55 (*O. floccosum*) y PcF2A29 (*O. piceae*).

Run	FIF1A55 *O. floccosum*			PcF2A29 *O. piceae*		
	Factor			Factor		
	Inoculum Size (C.F.U. ml^{-1})	Temperature (°C)	Agitation (r.p.m.)	Inoculum Size (C.F.U. ml^{-1})	Temperature (°C)	Agitation (r.p.m.)
1	$5{,}0x10^5$	26	200	$1{,}0x10^7$	26	100
2	$5{,}0x10^5$	26	100	$5{,}0x10^5$	18	100
3	$5{,}0x10^5$	18	200	$1{,}0x10^7$	18	200
4	$5{,}0x10^5$	18	100	$1{,}0x10^7$	18	100
5	$1{,}0x10^7$	26	200	$5{,}0x10^5$	18	200
6	$1{,}0x10^7$	26	100	$1{,}0x10^7$	26	200
7	$1{,}0x10^7$	18	200	$5{,}0x10^5$	26	200
8	$1{,}0x10^7$	18	100	$5{,}0x10^5$	26	100

Table 2. Effect of process variables and their interactions, shown as percentage of the total observed variability, on the response variables (yeast ratio and cellular concentration) during the albino FIF1A55 *O. floccosum* yeast-like growth experiment.

Cuadro 2. Efecto de las variables de proceso y sus interacciones, mostradas como porcentaje de la variabilidad total observada, en las variables respuesta (proporción de levaduras y concentración celular) durante el experimento de crecimiento levaduriforme de la cepa albina FIF1A55 *O. floccosum*.

Factor	Yeast ratio (%)			Concentration (C.F.U. ml^{-1})		
	24 h	48 h	72 h	24 h	48 h	72 h
A-Inoculum size (C.F.U. ml^{-1})	91,54	99,96	98,65	97,45	97,91	98,59
B-Temperature (ºC)	0,04	0,00	0,73	0,90	0,00	0,04
C-Agitation (r.p.m.)	4,57	0,00	0,13	0,09	0,27	0,13
AB	2,32	0,01	0,04	0,10	0,79	0,49
AC	0,06	0,01	0,00	1,02	0,36	0,05
BC	0,04	0,01	0,44	0,43	0,56	0,08
ABC	1,43	0,00	0,00	0,01	0,10	0,63
R^2	0,92	1,00	0,99	0,97	0,98	0,92
Adjusted R^2	0,90	1,00	0,98	0,97	0,98	0,90
Prediction R^2	0,85	1,00	0,98	0,95	0,96	0,85
Adequate precision	11,39	179,72	29,61	21,41	23,73	11,40

AB, AC, BC, ABC (interaction between factors A, B and C)

Table 3. Effect of process variables and their interactions, shown as percentage of the total observed variability, on the response variables (yeast ratio and cellular concentration) during the albino strain PcF2A29 *O. piceae* yeast-like growth experiment.

Cuadro 3. Efecto de las variables de proceso y sus interacciones, mostradas como porcentaje de la variabilidad total observada, en las variables respuesta (proporción de levaduras y concentración celular) durante el experimento de crecimiento levaduriforme de la cepa albina PcF2A29 *O. piceae*.

Factor	Yeast/Mycelium ratio (%)			Concentration (C.F.U. ml^{-1})		
	24 h	48 h	72 h	24 h	48 h	72 h
A-Inoculum size (C.F.U. ml^{-1})	40,03	88,25	80,95	98,26	93,77	95,77
B-Temperature (ºC)	34,03	4,91	0,02	0,73	0,4	0,58
C-Agitation (r.p.m.)	0,02	0,96	5,84	0,11	1,01	B
AB	23,51	2,59	7,83	0,15	0,5	1,63
AC	1,1	2,97	0,26	0,14	0,48	0,05
BC	0,01	0,07	1,49	0,11	3,81	1,77
ABC	1,3	0,25	3,61	0,5	0,02	0,16
R^2	0,98	0,88	0,81	0,98	0,94	0,96
Adjusted R^2	0,96	0,86	0,78	0,98	0,93	0,95
Prediction R^2	0,9	0,79	0,66	0,97	0,89	0,92
Adequate precision	15,61	9,49	7,14	26,05	13,44	16,48

AB, AC, BC, ABC (interaction between factors A, B and C)

Table 4. Treatments, variable levels and their results in the fermentation process for the *O. floccosum* albino strain.

Cuadro 4. Tratamientos, niveles de las variables y sus resultados en el proceso de fermentación de una cepa albina de *O. floccosum*.

Factor			Yeast (%)			Cell concentration (C.F.U. ml^{-1})		
Inoculum size (C.F.U. ml^{-1})	Temperature (C°)	Agitation (rpm)	24 (h)	48 (h)	72 (h)	24 (h)	48 (h)	72 (h)
$5,0x10^5$	18	100	1,5	5,2	13,5	$1,20x10^6$	$4,16x10^6$	$4,69x10^6$
$5,0x10^5$	18	200	4,0	3,8	15,0	$1,28x10^6$	$3,30x10^6$	$6,24x10^6$
$5,0x10^5$	26	100	5,3	7,3	22,6	$1,24x10^6$	$5,00x10^6$	$8,75x10^6$
$5,0x10^5$	26	200	1,9	12,2	15,2	$1,96x10^6$	$5,86x10^6$	$7,38x10^6$
$1,0x10^7$	18	100	59,2	81,6	90,5	$5,91x10^7$	$5,28x10^8$	$6,74x10^8$
$1,0x10^7$	18	200	27,0	84,4	89,4	$2,78x10^7$	$5,40x10^8$	$5,44x10^8$
$1,0x10^7$	26	100	71,8	89,8	94,0	$7,09x10^7$	$2,08x10^8$	$3,35x10^8$
$1,0x10^7$	26	200	56,3	89,7	86,8	$5,84x10^7$	$5,69x10^8$	$7,00x10^8$

Table 5. Treatments, variable levels and their results in the fermentation process of the *O. piceae* albino strain.

Cuadro 5. Tratamientos, niveles de las variables y sus resultados en el proceso de fermentación de una cepa albina de *O. piceae*.

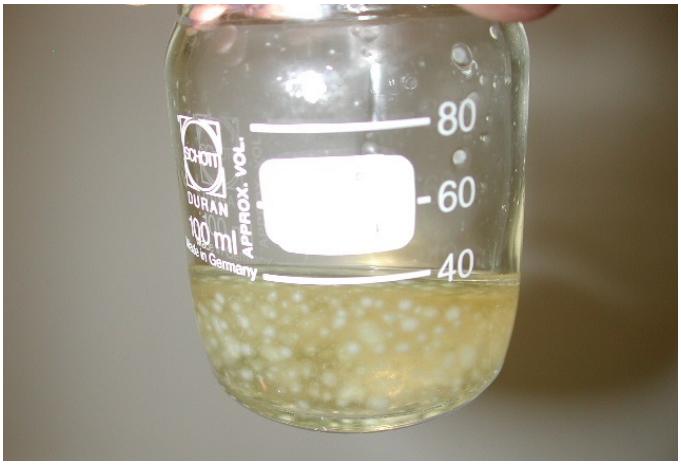
Factor			Yeast (%)			Cell concentration (C.F.U. ml^{-1})		
Inoculum size (C.F.U. ml^{-1})	Temperature (C°)	Agitation (rpm)	24 (h)	48 (h)	72 (h)	24 (h)	48 (h)	72 (h)
$5,0x10^5$	18	100	3,8	73,4	83,7	$1,64x10^6$	$9,19x10^6$	$4,69x10^7$
$5,0x10^5$	18	200	5,5	78,5	82,6	$1,36x10^6$	$8,76x10^6$	$4,04x10^7$
$5,0x10^5$	26	100	15,0	73,6	83,3	$1,25x10^6$	$2,48x10^6$	$1,95x10^7$
$5,0x10^5$	26	200	4,2	76,5	88,0	$1,19x10^6$	$1,07x10^7$	$2,91x10^7$
$1,0x10^7$	18	100	11,7	92,7	92,5	$2,79x10^7$	$5,45x10^8$	$6,93x10^8$
$1,0x10^7$	18	200	12,5	91,2	95,9	$7,84x10^7$	$2,60x10^8$	$4,15x10^8$
$1,0x10^7$	26	100	60,4	86,7	90,4	$4,48x10^7$	$2,35x10^8$	$4,84x10^8$
$1,0x10^7$	26	200	71,6	86,0	92,5	$2,09x10^7$	$6,40x10^8$	$8,05x10^8$

In the FIF1A55 *O. floccosum* albino strain cultures, the higher inoculum size (10^7 C.F.U. ml^{-1}) produced a larger proportion of yeast than the lower size (10^5 C.F.U. ml^{-1}) at all the evaluated times. At 48 hours of fermentation, the yeast proportion was more than 85% in treatments with a high inoculum size whereas the proportion of yeast in the low size inoculum was less than 23%. The effect of inoculum size is highly significant for inducing yeast growth in this species.

In addition, high inoculum size generated a larger concentration of spores per ml (>10^8 C.F.U. ml^{-1}), as compared to treatments with a low inoculum size, where only 10^6 cells per ml were obtained at 72 hours of fermentation. Likewise, high agitation velocity and temperature induced a greater quantity of spores ($7,0x10^8$ C.F.U. ml^{-1}) than other treatments where the concentration only reached 3,0 – 5,0 $x10^8$ C.F.U. ml^{-1} after 72 hours of fermentation (Table 4). Nevertheless, neither agitation velocity nor temperature produced a statistically significant effect.

An inoculum size less than 10^6 C.F.U. ml^{-1} in *O. floccosum* albino strains produced a high proportion of mycelium and favored the formation of mycelial structures having a “clumpy” appearance (Figure 1), which is the principal problem of this species in the yield of a fermentation process to produce spores on a semi-industrial scale. The formation of these morphological structures was not observed in cultures where the initial spore concentration was ≥ 10^7 C.F.U. ml^{-1}.

In microbiology, a biofilm can be described as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, besides they are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype (Harding et al. 2009). Based on this definition, the clumps formed in the cultures of *O. floccosum* could be considered as biofilms.



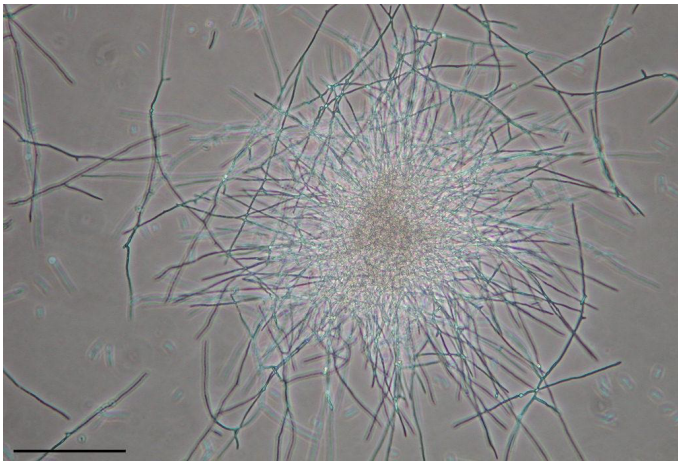


Figure 1. Liquid culture containing *O. floccosum* spores with less than 10^6 cells per ml **(a)** *O. floccosum* mycelial growth (arrow shown). **(b)** Micrograph of *O. floccosum* mycelia growth (200X. Bar length: 50 µm).

Figura 1. Cultivo líquido con esporas de *O. floccosum* con menos de 10^6 células por ml (a) Crecimiento micelial de *O. floccosum* (señalado con la flecha). (b) Microfotografía de crecimiento micelial de O. floccosum (200X. Longitud de la barra: 50 µm).

Condition	*O. floccosum*		*O. piceae*	
	Inoculum size			
	5,0x10^5 C.F.U.	1,0x10^7 C.F.U.	5,0x10^5 C.F.U.	1,0x10^7 C.F.U.
Inoculum				
T24h				
T48h				
T72h				

Figure 2. Phase-contrast micrographs of the effect of inoculum size during fermentation time in O. floccosum and O. piceae albino strain culture morphology in complex liquid medium. 400 X. Bar length: 10 µm.

Figura 2. Microfotografías de fase-contraste del efecto del tamaño del inóculo a diferentes tiempos de fermentación en la morfología de cultivos de cepas albinas de O. floccosum y O. piceae en medio de cultivo líquido complejo. 400X. Longitud de la barra 10 µm.

Table 5 shows the values for the relationship for yeast proportion and spore concentration obtained with the different treatments at three fermentation times for the albino strain PcF2A29.

For the albino strain PcF2A29 (*O. piceae*), a low inoculum size ($5{,}0x10^5$ C.F.U. ml^{-1}) resulted in a high proportion of mycelium at 24 hours (less than 15% of yeast). However, at 48 hours the average percentage of yeast was 75% and at 72 hours the proportion of fermentation reached 84% (Table 5). This behavior suggests the existence of a different mechanism for the effect of inoculum size on the morphology of the albino strains for the species *O. piceae* in comparison to the species *O. floccosum*, where a low inoculums size always generated a low proportion of yeast-like morphology of the culture.

In the case of the strain PcF2A29 of *O. piceae*, together with the inoculum, the temperature and the interaction between the inoculum size-temperature were significant factors in the yeast/mycelium ratio.

A higher inoculum size produced a larger quantity of spores per ml (on the order of 10^8), while an initial concentration of $5{,}0x10^5$ only induced a final spore concentration on the order of 10^7 (Table 5). *O. piceae* produced a larger quantity of spores than *O. floccosum* in all the evaluated treatments indicating that *O. piceae* is capable of generating a higher concentration of yeast at a low inoculum size as shown in Figure 2.

Discusion

Preliminary experiments determined that at 24 hours of fermentation more than 70% of the total observed variability in the yeast growth in albino strains of *O. floccosum* and *O. piceae* was principally due to factors such as inoculum size, temperature and their interaction (Berrocal et al. 2011).

The results obtained in this study demonstrate the relative importance of inoculum size in the control of dimorphism for albino strains of the genus Ophiostoma. Indeed, the effect of inoculum size has been widely described for other species (Kulkarni and Nickerson, 1981; Nickerson et al., 1982; Muthukamar and Nickerson, 1984; Muthukamar et al., 1985; Jensen et al., 1992; Hornby et al., 2004; Nickerson et al., 2006). These studies found that dimorphism of the fungi *O. ulmi* (*Ceratocystis ulmi*), *Candida albicans*, *Histoplasma capsulatum* and *Mucor rouxii,* was dependent on inoculum size (Kulkarni, 1981).

Hornby et al. (2004) determined that low inoculum size ($4{,}0x10^5$ C.F.U. ml^{-1}) produced a 5% yeast proportion at 24 hours of fermentation in *O. ulmi* cultures grown in a media containing glucose, phosphates and L-proline as nitrogen source, whereas a high size inoculum ($\geq 10^6$) resulted in a yeast proportion greater than 85%. M^{c}Neel (1983) found the same correlation between the initial concentration of cells and morphology, where inoculum densities greater than $1{,}0x10^6$ consistently favored yeast growth in *O. ulmi* in defined liquid culture media. Besides, in *Candida albicans*, a high inoculum size produced a yeast/mycelium ratio between 65 and 100% at 24 hours of growth with different strains of the species and various liquid culture media (Hornby et al. 2001).

The effect of temperature on dimorphism has been carefully analyzed in pathogenic human fungi. The microorganisms *Histoplasma capsulatum*, *Blastomyces dermatitides*, *Paracoccidiodes brasiliesis*, *Sporothrix schenckii* and *Coccidioides immitis* exhibit a dimorphic growth associated to this factor. In all cases, the mycelial phase appears at low temperatures (20-25 °C), while yeast dominance occurs at 37 °C (Nguyen and Sil, 2008, Cano and de Aguiar, 1991 and Deacon, 2006).

According to Deacon (2006), temperature induces a signaling effect on culture growth morphology that can also be extrapolated to albino fungus from the genus *Ophiostoma*. Based on our results for these fungi, whose optimal growth temperature is between 20-25 °C, we can suggest that lower temperatures (18 °C) would produce a condition of stress that would influence the growth of mycelium as a response to the environmental conditions.

Conclusions

The inoculum size factor had the major influence in promoting yeast growth in the albino strains PcF2A29 (*O. floccosum*) and FIF1A55 (*O. piceae*). An inoculum size of $1{,}0x10^7$ resulted in the highest yeast/mycelium ratio as well as a higher concentration of spores (yeast) as compared to the inoculum size of $5{,}0x10^5$ C.F.U. ml^{-1} after 72 hours of fermentation. Temperature was a significant factor only in yeast/mycelium ratio at 24 h of fermentation in the albino strain from the species *O. piceae*. For that reason, the conditions that favored the generation of cultures with a large proportion of yeast are: an inoculum size of $1{,}0x10^7$ ml^{-1}, temperature at 25 °C, and a rotation speed between 100-150 rpm. The results of this work demonstrate the existence of factors that directly influence the dimorphic growth of albino strains from the genus Ophiostoma. Reproducible control of these factors will help to improve the yeast like spores production process of these kind of fungi.

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