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ORIGINAL ARTICLE

Effect of farnesol on *Candida dubliniensis* morphogenesis

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Candida albicans, *Candida dubliniensis*, farnesol, hyphae, morphology alteration, pseudohyphae.

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Abstract

Aims: Cell–cell signalling in *Candida albicans* is a known phenomenon and farnesol was identified as a quorum sensing molecule determining the yeast morphology. The aim of this work was to verify if farnesol had a similar effect on *Candida dubliniensis*, highlighting the effect of farnesol on *Candida* spp. morphogenesis.
Methods and Results: Two different strains of *C. dubliniensis* and one of *C. albicans* were grown both in RPMI 1640 and in serum in the presence of absence of farnesol. At 150 μmol l^{-1} farnesol the growth rate of both *Candida* species was not affected. On the contrary, farnesol inhibited hyphae and pseudohyphae formation in *C. dubliniensis*.
Conclusion: Farnesol seems to mediate cell morphology in both *Candida* species.
Significance and Impact of the Study: The effect of farnesol on *C. dubliniensis* morphology was not reported previously.

Introduction

Quorum sensing is the ability of micro-organisms to communicate, via signalling molecules, in order to coordinate their behaviour. As the discovery of a substance produced by *Vibrio fischeri* which induced bioluminescence at the early growth stage (Nealson *et al.* 1970), the quorum sensing phenomenon in bacteria has been fully studied. However, concerning yeast only few studies have been performed. In fact, for *Candida* until now this regulatory mechanism has only been demonstrated in *C. albicans* (Hornby *et al.* 2001; Ramage *et al.* 2002; Chen *et al.* 2004), in *C. tropicalis* (Hazen and Cutler 1979) and recently in *C. parapsilosis* (Laffey and Butler 2005).

For *C. albicans*, some compounds were already described as quorum sensing molecules (QSM), namely, tyrosol (Chen *et al.* 2004) and farnesol (Hornby *et al.* 2001). Specifically, farnesol was first identified by Hornby *et al.* (2001) as QSM excreted by *C. albicans* that was found to inhibit the yeast-to-mycelium conversion without interference on cell growth. Although the role of farnesol in the pathogenicity of *C. albicans* is poorly understood there is indirect evidence that farnesol increases the virulence of this fungus (Shea and Del Poeta 2006). The mechanisms by which farnesol mediates its effect is also not known. Deletion of the histidine kinase *CHK1*, a component of a stress-adaptation signalling pathway, was found to protect *C. albicans* from the inhibitory effects of farnesol on hyphal transition and biofilm formation (Kruppa *et al.* 2004). Shea and Del Poeta (2006) suggested that farnesol could influence *C. albicans* biology in part by modulating the sterol synthesis pathway.

Currently, the emergence of Non-*C. albicans Candida* (NCAC) species is becoming relevant. One of the pathogenic NCAC species is *C. dubliniensis* that was recovered from oral cavities of HIV-infected patients (Sullivan *et al.* 1995). Despite the lower prevalence of *C. dubliniensis* in blood cultures, once an infection has been established this species can result in increased morbidity (Chan-Tack 2005). This species has been misidentified with *C. albicans* for years mainly because of their capacity to form mycelia (Sullivan *et al.* 2005).

One of the major determinants of *Candida* virulence is its ability to form hyphae or pseudohyphae from unicellular yeast (Yang 2003). Thus, it is important to get insights into the factors that regulate the morphologic transition. The correlation between dimorphism and pathogenecity suggests that interfering with the morphological

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switch could be an attractive method for controlling pathogenecity. Manipulating dimorphism could force an opportunistic pathogen to exist only in a form that should not damage the host (Nickerson *et al.* 2006). As farnesol acts preventing the formation of the more pathogenic *C. albicans* form, hyphae, it might be useful as a novel therapeutical antifungal compound for immunocompromised patients since it would not upset the natural resident flora (Hornby *et al.* 2001). Accordingly, the main aim of this work was the investigation of the effect of QSM, namely farnesol, on the morphology of *C. dubliniensis*.

Materials and methods

Strains

Two strains of *Candida dubliniensis* (CBS 7987 and CBS 7988, from Centraalbureau voor Schimmelcultures, Germany) and one strain of *Candida albicans* (CECT 1472, from 'Colección Española de Cultivos Tipo', Spain) were used in this study. For all the assays, these strains were grown for 24 h in Sabouraud dextrose agar at 37°C. Then, batches of yeast cells were cultured in SDB for 24 h at 37°C and 130 rev min^{-1}. Under these conditions both *Candida* species grew in the yeast form.

Media and chemicals

In the current experiments, two different media were used to favour filamentation: RPMI 1640 medium (Sigma, Sintra, Portugal) buffered with morpholinepropanesulfonic acid (MOPS – Sigma) and serum (Foetal Bovine Serum; Gibco, Barcelona, Spain) 10% in water (Gilfillan *et al.* 1998). RMPI was used as a common *Candida* growth medium and serum was selected for inducing *C. dubliniensis* hyphae formation. Farnesol (trans, trans farnesol – Sigma) was prepared diluting the solution supplied in methanol to a stock solution concentration of 600 mmol l^{-1} and stored at −20°C. Working solutions were prepared in the different filamentation media at final concentrations of 300, 150, 15 and 1·5 μmol l^{-1}.

Effect of farnesol on growth and cell morphology

After the preinoculum in SDB, cells were harvested by centrifugation (1663 ***g*** for 15 min, 4°C) and washed twice with sterile ultrapure water. Cellular density was adjusted to 1×10^6 cell ml^{-1} using a Neubauer counting chamber. These standardized cell suspensions were prepared in the two filamentation media that were supplemented with different farnesol concentrations. For each experiment, a farnesol free control was used.

In order to evaluate the effect of farnesol on growth rate of batch cultures (grown at 37°C and 130 rev min^{-1}), samples were collected at selected times (2, 4, 6, 8, 10, 12, 14 and 18 h) and cell density was assessed by optical density (620 nm) in a spectrophotometer [Synergy HT-I (BIO-TEK, Winooski, VT, USA)].

To evaluate the effect of farnesol on cell morphology, cell suspensions were grown for 12 h and both macroscopic and microscopic evaluations were performed. Phase contrast microscopic counts were carried out on a Zeiss Axioskop microscope fitted with 10× and 40× objectives and equipped with a Carl Zeiss AxioCam (HR/MR; Cambridge, UK). Cellular morphology was characterized according to Sudbery *et al.* (2004) into yeast, pseudohyphae and hyphae. The quantitative assessment of farnesol on cellular morphology was performed by calculating the percentage of hyphae or pseudohyphae inhibition (% I) based in the number of hyphae or pseudohyphae in the presence ($HP_{presfarn}$) and absence ($HP_{absfarn}$) of farnesol [eqn (1); Chiller *et al.* 2000].

$$\%\,I = \frac{HP_{absfarn} - HP_{presfarn}}{HP_{absfarn}} \times 100 \qquad (1)$$

All experiments were repeated three times with duplicate samples, and 15 fields per slide were examined.

Statistical analysis

The resulting data were analysed using SPSS (Statistical Package for the Social Sciences, Chicago, IL, USA). One-way ANOVA with Bonferroni test was used to compare either the strains response or the different concentrations assayed. All tests were performed with a confidence level of 95%.

Results

Farnesol and *Candida* growth

As a prerequisite for its quorum sensing activity, farnesol must not alter the yeast growth rate (Hornby *et al.* 2001). The evaluation of the influence of farnesol on cell growth and growth rate was determined following growth of *C. dubliniensis* 7987, *C. dubliniensis* 7988 and *C. albicans* 1472 in 150 μ mol l^{-1} of farnesol in RPMI 1640 along the time and comparing with the control profile (RPMI 1640). Figure 1 represents the growth rate of *C. dubliniensis* 7987 either in the presence or absence of farnesol. Similar results were obtained for the other strains (data not shown).

Farnesol concentrations of 150 μmol l^{-1} do not have significant inhibitory effect ($P < 0{\cdot}05$) on *C. dubliniensis*

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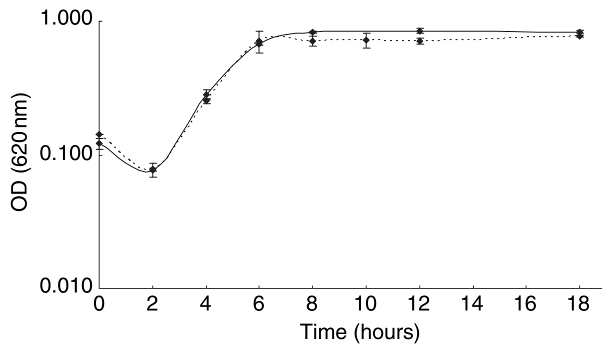

Figure 1 Growth curves of *C. dubliniensis* 7987 grown in RPMI 1640 (—) and in RPMI 1640 supplemented with 150 μmol l^{-1} farnesol (- - - -).

growth and growth rate (Fig. 1). It was also confirmed that farnesol did not affect *C. albicans* growth and growth rate (data not shown) as it was previously reported for other strains (Hornby *et al.* 2001). Although *C. dubliniensis* lag phase is higher than for *C. albicans*, the growth profile or growth rate of both species is not altered by the presence of farnesol. For lower farnesol concentrations (1·5 and 15 μmol l^{-1}), there were also no differences between the growth in presence of farnesol and the control (data not shown).

Farnesol and *Candida* morphology in RPMI 1640

To evaluate the activity of farnesol on *Candida* morphology, samples from batch cultures with and without farnesol (150 μmol l^{-1}) treatment were collected at stationary phase (12 h) and observed under phase contrast microscopy (Fig. 2).

Both *C. dubliniensis* and *C. albicans* grow in yeast form in SDB (the medium used for the preinoculum; Fig. 2i). At low cell population density (1×10^6 cells ml^{-1}), *C. albicans* formed true hyphae in RPMI 1640, as it was expected (Fig. 2c-ii), while *C. dubliniensis* strains grow as pseudohyphae in this medium (Figs 2a-ii,b-ii). However, if any of the cells suspensions

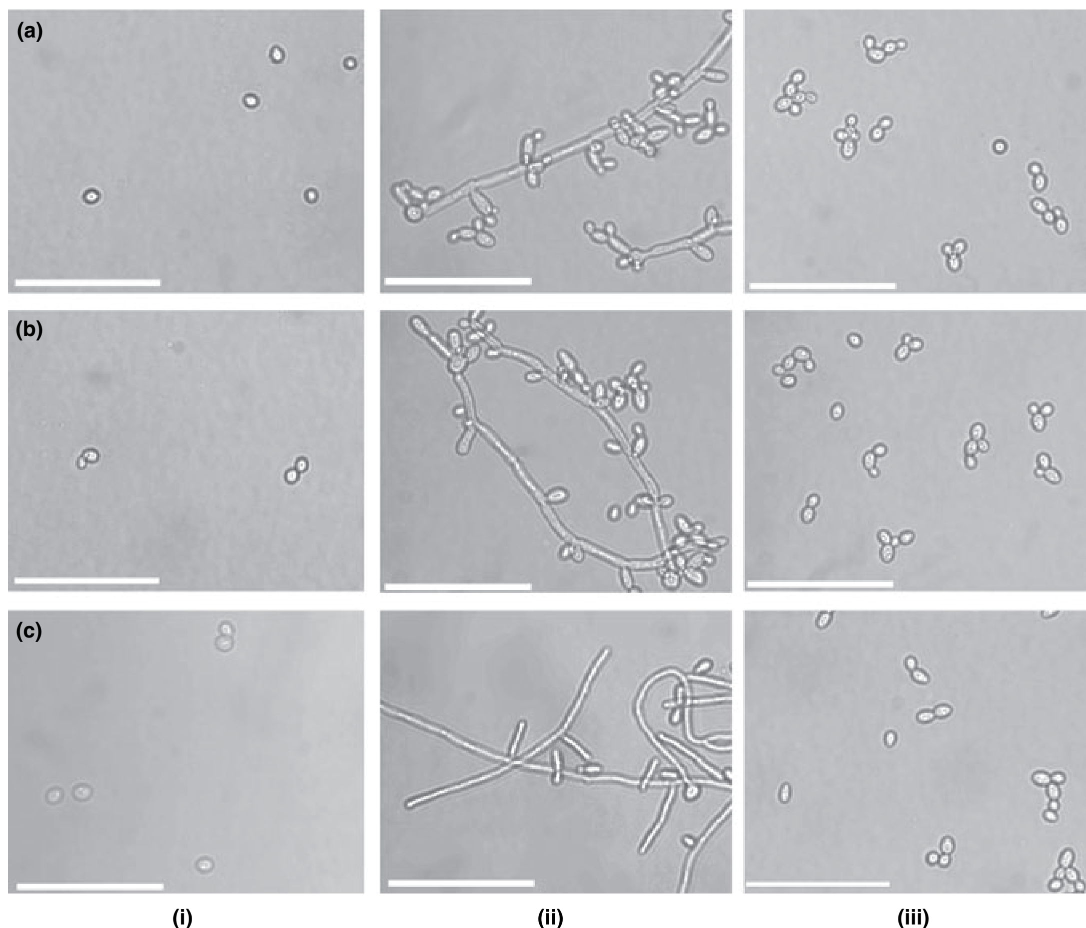

Figure 2 Phase contrast microscopy observation of (a) *C. dubliniensis* 7987, (b) *C. dubliniensis* 7988 and (c) *C. albicans* 1472 grown for 12 h in SDB (i), in RPMI 1640 (ii) and in RPMI 1640 supplemented with 150 μmol l^{-1} of farnesol (iii). The bar in the images corresponds to 50 μm.

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Table 1 Percentage of hyphae inhibition in *C. albicans* 1472, when grown for 12 h in the presence of 1·5, 15 and 150 μmol l^{-1} of farnesol in RPMI 1640

	Farnesol concentration (μmol l^{-1})		
	1·5	15	150
% hyphae inhibition (average ± SD)	73·5 ± 16·8	82·8 ± 10·5	97·4 ± 3·8

is treated with 150 μmol l^{-1} farnesol (Fig. 2iii) occur the inhibition of the filamentous form with high prevalence of blastospores.

According to other reports (Kim *et al.* 2002; Ramage *et al.* 2002) it would be expected that in *C. albicans* different farnesol concentrations triggered different effects on cellular morphology. Therefore, to confirm and quantitatively rank the sensitivity of *Candida* yeast cells to farnesol, cells suspensions were cultured for 12 h in different concentrations of farnesol and the percentage of inhibition of hyphae (Table 1) and pseudohyphae (Fig. 3) formation was determined.

Figure 3 and Table 1 show that farnesol concentrations assayed are effective on both *C. albicans* and *C. dubliniensis* morphology inhibiting the formation of hyphae and pseudohyphae, respectively. For all the concentrations tested, it can be noticed that no significant differences were observed between the percentage of inhibition of hyphae/pseudohyphae formation among the strains ($P > 0·005$). However, for all the strains, cells treated with the lower farnesol concentrations (1·5 and 15 μmol l^{-1}) presented significant differences ($P < 0·05$) when compared with cells treated with the highest farnesol concentration (150 μmol l^{-1}), showing some weak dose dependence. It is interesting to note that even in the presence of 1·5 μmol l^{-1} farnesol the percentage of inhibition of either hyphae or pseudohyphae was higher than 50%. For the higher farnesol dosage (150 μmol l^{-1}) 90% of inhibition was achieved. It can also be referred that 300 μmol l^{-1} of farnesol inhibits the complete formation of hyphae and pseudohyphae without exhibiting any inhibition effect upon cellular growth (data not shown). Although both species grow with different morphologies in RPMI 1640, farnesol blocks the yeast to hyphae/pseudohyphae conversion in all strains.

Farnesol and *Candida* morphology in serum

The effectiveness of farnesol in inhibiting hyphae formation on *C. dubliniensis* was also assessed in a germ tube inducing medium, Foetal Bovine Serum (Gilfillan *et al.* 1998; Fig. 4). The results showed that *C. dubliniensis* form true hyphae in the presence of 10% serum (Fig. 4a). On the contrary, the total absence of hyphae in cells treated with 150 μmol l^{-1} farnesol (Fig. 4b) proves the farnesol effectiveness on *C. dubliniensis* morphology.

In fact, in the conditions tested for both strains of *C. dubliniensis* 100% inhibition was achieved not only by 300 μmol l^{-1} of farnesol but also with 150 μmol l^{-1}. Interestingly, for the strain of *C. albicans* studied the dose of farnesol that inhibits 100% of hyphae formation is higher than for *C. dubliniensis*, being even higher than 400 μmol l^{-1} (results not shown). The results show that in *C. dubliniensis* farnesol not only inhibits pseudohyphae (in RPMI 1640) but also suppresses hyphae formation.

Discussion

Farnesol was found to be excreted by *C. albicans* (Hornby *et al.* 2001) and to act as a QSM regulating its morphology. To the authors knowledge, there are no studies concerning the effect of this molecule on *C. dubliniensis* growth and morphology, which seems important once this species is closely related to *C. albicans* and hyphae

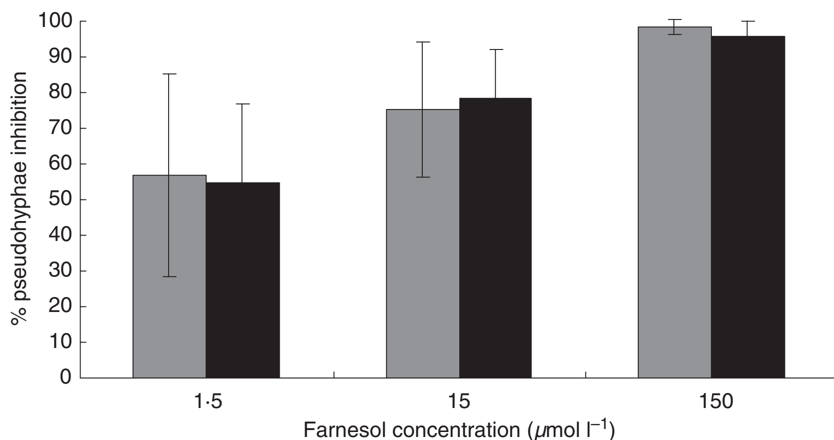

Figure 3 Percentage of pseudohyphae inhibition in *C. dubliniensis* 7987 (■) and *C. dubliniensis* 7988 (■), when grown for 12 h in the presence of 1·5, 15 and 150 μmol l^{-1} of farnesol in RPMI 1640.

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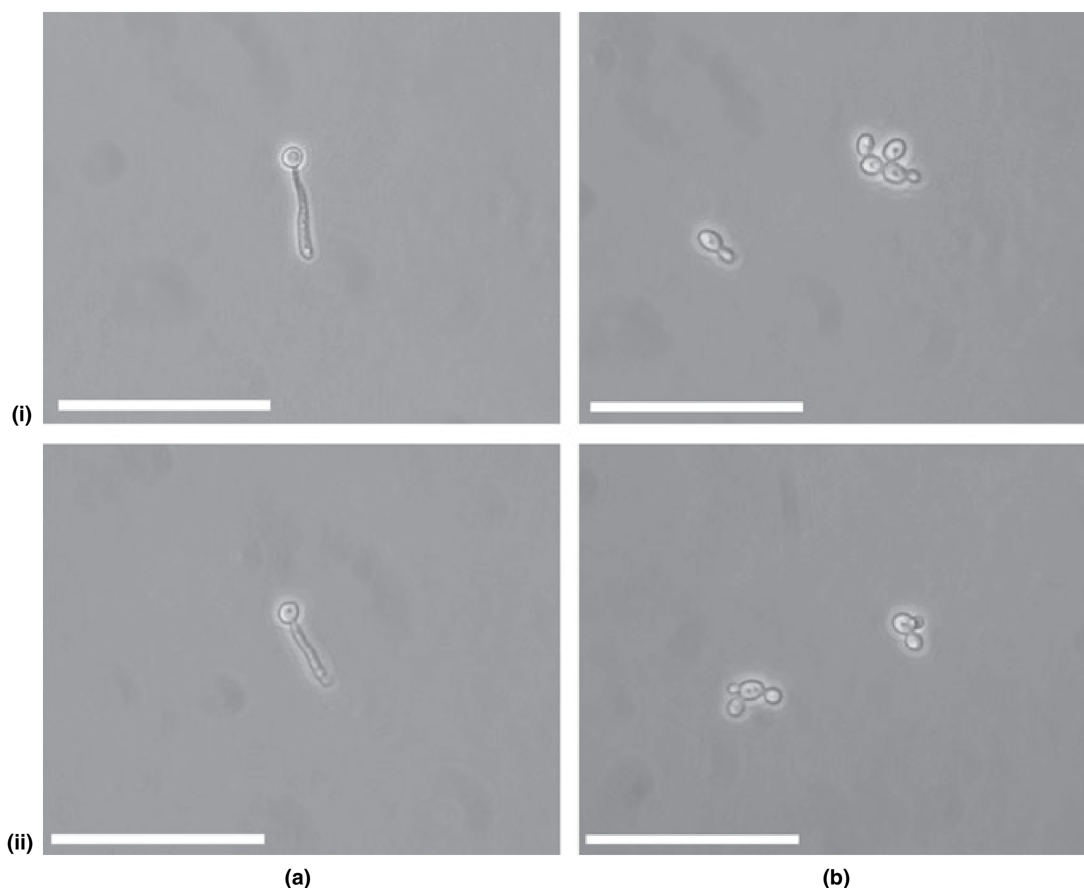

Figure 4 Phase contrast microscopy observation of (i) *C. dubliniensis* 7987 and (ii) *C. dubliniensis* 7988 grown in 10% serum in the absence (a) or presence (b) of farnesol 150 μmol l^{-1} in 1 h batch cultures. The bar in the images corresponds to 50 μm.

formation is an important virulence factor. The results presented herein show that farnesol affects *C. dubliniensis* morphology, blocking not only hyphae but also pseudohyphae development. The effect of (*E,E*)-farnesol was evaluated concerning cellular and filamentous growth. This farnesol isomer was chosen according to the results reported in the literature, which show that it is the most active isomer against the *C. albicans* morphological transition (Shchepin *et al.* 2003).

According to the results obtained, the growth of both *C. dubliniensis* strains was not inhibited by the presence of 150 μmol l^{-1} farnesol (Fig. 1). Moreover, the results also corroborate, what was already found by other authors (Hornby *et al.* 2001; Ramage *et al.* 2002), that farnesol does not affect *C. albicans* growth rate. Thus, in terms of growth rate, farnesol does not have any effect over both species, in the concentrations assayed. However, Kim *et al.* (2002) showed a growth inhibition of 35% for *C. albicans* grown in the presence of farnesol 450 μmol l^{-1}. In this sense, it cannot be excluded that for *C. dubliniensis* similar effect could be observed for higher farnesol concentrations. However, the mechanism involved in this growth inhibition is not yet well known. Nevertheless, farnesol concentrations of 25 μmol l^{-1} and 10 μmol l^{-1} applied externally to *Saccharomyces cerevisiae* (Machida *et al.* 1998) and *Aspergillus nidulans* (Semighini *et al.* 2005), respectively, led to growth inhibition through induced intracellular reactive oxygen species production. Altogether, these results show an antagonistic effect of farnesol among different fungi species suggesting that, farnesol may contribute to the success of *Candida* spp. as pathogen.

Considering filamentous growth, different concentrations of farnesol supplemented to both RPMI 1640 and 10% serum media were used in order to verify the dose effect on morphology inhibition. In RPMI 1640, the behaviour of the different strains tested was similar, showing an inhibition of hyphae/pseudohyphae *c.* 90% for the higher farnesol concentrations and higher than 50% for the lower farnesol concentration assayed (1·5 μmol l^{-1}; Fig. 3 and Table 1), indicating some dose dependent effect. The lower farnesol concentration

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to achieve 50% inhibition of hyphae/pseudohyphae formation may have a relevant physiological effect since several *C. albicans* strains grown for 24 h produce farnesol at 2–4 μmol l^{-1} (Hornby and Nickerson 2004). To date, there are no reports on the literature concerning the production of farnesol by *C. dubliniensis* planktonic or biofilm growing cells. However, it has been shown in competition growth assays performed with planktonic and biofilm cells that *C. albicans* had competitive advantage over *C. dubliniensis* (Kirkpatrick *et al.* 2000). Whether this interspecies interaction is mediated by an environmental factor such as farnesol would be worth to investigate.

In RPMI 1640, a medium where *C. albicans* grows in hyphal form, *C. dubliniensis* formed pseudohyphae (Fig. 2ii), which could be observed after 2 h of growth (data not shown). On account of this, a germ tube formation medium was used to evaluate the effect of farnesol on *C. dubliniensis* hyphae development. The selected medium was serum based on studies of Sudbery *et al.* (2004). In fact, the results in serum (Fig. 4), were in agreement with the ones obtained in RPMI 1640 (Fig. 3), with farnesol inhibiting yeast to hyphae transition in *C. dubliniensis* strains. As suggested by Mosel *et al.* (2005) the higher concentrations of farnesol needed to suppress hyphae formation in serum are due to nonspecific lipid binding abilities of the albumins in that medium. Moreover, the results presented show that for the same farnesol concentrations hyphae inhibition in *C. dubliniensis* strains was more effective than for *C. albicans*.

The role of farnesol in the pathogenicity of *Candida* spp. is not well understood. Although the initial studies with farnesol, as QSM, pointed out its use as a therapeutic agent, recent reports show that fluconazole pretreated *C. albicans* cells, producing significantly higher levels of farnesol, presented enhanced pathogenicity (Navarathna *et al.* 2005). Interestingly, it has been described that *C. dubliniensis* exposed to fluconazole present higher adherence to epithelial cells than yeast cells that had not been exposed to the drug (Zepelin *et al.* 2002). Considering all these findings it should be evaluated if exogenous and endogenous sources of farnesol, plays or not a pivotal role in the adherence of *C. dubliniensis* cells contributing to its pathogenicity.

Farnesol has been shown for the first time to inhibit the formation of both pseudohyphae and hyphae in two strains of *C. dubliniensis*. Although *C. dubliniensis* has the capacity to produce hyphae, several studies (Sullivan *et al.* 2005) pointed out that the control of morphogenesis in *C. albicans* and *C. dubliniensis* may have different regulation pathways. In spite of these observations, the results presented herein show that farnesol controls the morphogenesis of *C. dubliniensis* in a similar way to that of *C. albicans*. In fact, quorum sensing mediated throughout farnesol seems to be a phenomenon used by *Candida* spp. to regulate its morphogenesis. However, the role of farnesol in *C. dubliniensis* virulence and interaction with other micro-organisms, as well as the molecular mechanism and signal pathways involved in the action of farnesol should be further understood.

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