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Full Length Research Paper

Lithium chloride affects mycelial growth of white rot fungi: Fungal screening for Li-enrichment

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The enrichment of edible mushrooms with lithium (Li) may be a strategy to provide forms of lithium that are more soluble and bioavailable for humans. Therefore, it is important to determine which species of fungi are able to grow in the presence of Li, and which concentrations of Li allow fungal growth. Twelve white rot fungi strains were grown in potato dextrose agar media, supplemented with 0 to 1.65 g L^{-1} lithium chloride (LiCl). The fungal growth rate, morphological alterations of the colonies, changes in the length of the lag phase, fungal dry mass, changes in hyphae diameter and cell length were evaluated. Most fungi had decrease in their growth rates and dry mass, and had macroscopic/microscopic morphological alterations at increasing LiCl concentration. Generally, the fungi were sensitive to LiCl. However, *Pholiota nameko* was moderately tolerant to LiCl and *Pleurotus ostreatusroseus* tolerate the highest LiCl level tested, suggesting that it is the most appropriate fungus for Li-enrichment.

Key words: Fungal enrichment, mycelial morphology, screening.

INTRODUCTION

Lithium has an irregular distribution in the Earth's crust (Rybakowski, 1995; Aral and Vecchio-Sadus, 2008). As a result, some populations have a low dietary lithium intake (Rybakowski, 1995). Low levels of lithium in the blood have been related to the occurrence of some psychiatric disorders, (Schrauzer and Shrestha, 1990; Severus et al., 2009) and lithium compounds such as, lithium carbonate, are commonly used to treat bipolar disorder (Rapoport et al., 2009). It has been shown that the rates of rape and homicide are higher in counties with low levels of lithium in drinking water supplies (Dawson et al., 1970). The development of foods with high lithium availability could be a way to increase lithium intake.

Some Basidiomycetes are able to absorb and accumulate minerals, such as lithium, in mushrooms (Widmer, 1999; Bayramoglu et al., 2002; Figlas et al., 2010; de Assunção et al., 2012). The highest lithium content of wild mushroom was 12 mg kg^{-1} found in *Thelephora vialis* (Yin et al., 2012). However, the lithium content found in wild mushroom seems to vary among different fungi species and regions (Vetter, 2005; Yin et al., 2012; Falandysz and Borovička, 2013). The content

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of lithium in mushrooms found in China (Yin et al., 2012) were higher than values found in Hungary and Italy (Vetter, 2005; Giannaccini et al., 2012). This could be due to a higher lithium concentration in China soil than Hungary and Italy soils. Indeed, de Assunção et al. (2012) showed that the lithium levels found in the Li-enriched mushrooms were proportional to the lithium levels found in the substrate. These authors also showed that the enrichment of substrate with a soluble lithium salt (LiCl), do not affect the biological efficiency (productivity) of *Pleurotus ostreatus.*

Lithium is toxic, depending on the concentration, to animals, plants and microorganism (Aral and Vecchio-Sadus, 2008) which can be limitant for fungal Li-enrichment. Richter et al. (2008) tested the growth of 40 species of fungi in culture media with 1.5, 3 and 6 g L^{-1} of LiCl and observed that the growth rate of most of the fungi greatly decreased at 3 g L^{-1} of LiCl and above. The white-rot fungus, *P. ostreatus*, decreased its growth rate by 90% in 1.5 g L^{-1} of LiCl and its growth was inhibited at higher concentrations of LiCl (Richter et al., 2008). Wildman (1991) also observed that culture media with 6 g L^{-1} of LiCl inhibited *Trichoderma* spp. growth. As the sensitivity or tolerance of fungi to LiCl varies considerably, it is important to determine the concentration of this salt that allows mycelial growth and development. This will allow us to choose the fungi species more appropriate for fungal Li-enrichment.

MATERIALS AND METHODS

Microorganism

All the fungi species chose for the screening are cultivated at industrial scale. We also selected only fungi that produce mushrooms on non-composted substrate because this procedure is easier and cheaper than on composted substrates. The fungi used were *P. ostreatus* (PLO 06), *P. ostreatus* (P.98), *Pholiota nameko* (PH 01), *Pleurotus ostreatusroseus* (PLO 13), *Pleurotus citrinopiliatus* (PLO A), *Grifolla* sp. (GF), *Grifolla frondosa* (GF-JP), *Ganoderma subamboinense* var. *laevisporum* (GR 117), *Pleurotus eryngii* (PLE 04), *Hericium herinaceum* (HE 01), *Lyophillum shimeji* (LY 01) and *Lentinula edodes* (UFV 73). All these fungi belong to the collection of the Laboratório de Associações Micorrízicas, Departamento de Microbiologia, BIOAGRO, UFV. Two isolates of *P. ostreatus* were included by presenting different commercially important characteristics, such as white cap by PLO 06 and the light brown cap by P.98. Mushrooms of these species are sources of protein, fiber, vitamins and minerals (Barros et al., 2008; de Assunção et al., 2012). We incubated these fungi in potato dextrose agar (PDA, Fluka Analytical, St. Loius, Missouri, USA) at 22 ± 1°C for seven days.

Culture media and cultivation conditions

Fungi were grown on PDA containing 0, 0.30, 0.60, 0.90, 1.20 or 1.65 g L^{-1} of LiCl. This salt of lithium has previous been used for Li-enrichment of mushrooms (de Assunção et al., 2012). The culture medium was autoclaved at 121°C for 20 min. Plugs of inoculum 5 mm in diameter were cut from the perimeter of an actively growing fungus colony. Inoculum plugs were firmly placed with the mycelium side down in the center of the experimental plates. Six replicate plates were prepared for each LiCl concentration and fungus, and were then incubated at 22 ± 1°C.

Lag phase and growth rate

After incubation, the colonies were observed daily to determine the start of mycelial growth. The fungal growth rate was determined by measuring the colony's diameter in two directions that were perpendicular to each other. Measurements were made for 45 days or until maximum Petri dish colonization. The measurements were made every 48 h for fungi with high growth rates (PH01, PLO 06, P.98, PLO 13, PLO A, PLE 04, GR 117) and every 72 h for fungi with low growth rates (GF, GF-JP, UFV 73, HE and LY 01).

Colony morphology

The morphological characteristics of colonies were qualitatively evaluated using the following criteria: a) medium color alteration, b) colony color change, c) mycelial density decrease and d) colony growth appearance (uniform or uneven).

Hyphae diameter and septa distance

For diameter and septa distance measurements, we used epifluorescence microscopy to observe fungus samples stained with calcofluor. To standardize the measurements, the samples that were used were all young hyphae (hyphae in the border of coverslips). Photos were taken with a digital camera FUJIX HC-300Z and then they were processed with the software Image Pro Plus. For septa distance measurements, we used the photos 400x amplified and for hyphae diameter, we used the photos 1000x amplified. Measurements using photos more amplified are more precise and accurate. All the measurements were made using the software Image Pro Plus. These measurements were made because some fungi alter hyphae morphology under inappropriate environmental conditions and because the microscopic appearance can aid understanding how the fungi adapt to environments with LiCl.

Biomass

To determine the mycelial dry mass, the entire contents of Petri dish (mycelium + culture media) were put in a bottle with distilled water and heated in a water bath (1 to 5 min) to dissolve all culture medium. Then, the solution was filtered and the mycelium was dried in an oven at 80°C until a constant weight was reached.

Statistical analysis

The experiment used a randomized design. The data, except for the macroscopic morphological data, were subjected to analysis of variance, and the averages were compared by Tukey's test ($p < 0.05$) using Saeg software (version 9.1, Universidade Federal de Viçosa).

RESULTS

The fungi were affected differently by the LiCl. Among the

fungi assayed, five strains increased the lag phase (Figure 1). The *H. herinaceum* (HE 01) and *L. shimeji* (LY 01) strains showed a 4- to 5-fold increase in the lag phase at 0.3 g L^{-1} LICl ($P < 0.001$). Strains of both *Grifolla* (GF and GF-JP; $P < 0.001$) and *P. ostreatus* (PLO 06; $P < 0.001$) increased their lag phases at 0.60 and 0.90 g L^{-1} LICl, respectively; however, this increase was only up to 3-fold. The increase in the lag phase at low concentrations of LiCl indicated that *H. herinaceum*, *L. shimeji* and both strains of *Grifolla* are sensitive to LiCl.

The addition of LiCl to the culture media reduced the biomass and growth rates of most fungi (Figures 2 and 3). *P. ostreatus* (PLO 06; $P < 0.001$), *Lentinula edodes* (UFV 73; $P < 0.001$), *H. herinaceum* (HE 01; $P < 0.001$), *L. shimeji* (LY 01; $P < 0.001$), *P. eryngii* (PLE 04; $P < 0.001$) and *P. ostreatus* (P.98; $P < 0.001$) had their growth rates decreased at 0.30 g L^{-1} of LiCl and, for the majority of fungi, this reduction occurred at 0.60 g L^{-1} of LiCl. Ten strains had their growth inhibited by LiCl. Only *P. nameko* (PH 01) and *P. ostreatusroseus* (PLO 13) were able to grow in media enriched with 1.65 g L^{-1} of lithium (Figure 2). Biomass production (Figure 3) seemed to be a parameter more sensitive to LiCl than the growth rate (Figure 2), although sometimes they responded similarly. For *P. citrinopiliatus* (PLO A) and *P. eryngii* (PLE 04), the biomass decreased more than the growth rate. The biomass of *P. nameko* was completely different from the growth rate. While small changes in the growth rate were observed, the biomass began to decrease at 1.20 g L^{-1} LiCl and reduced by 90.4% at the highest level of LiCl tested. Overall, the majority of fungi tested were inhibited by LiCl. Considering the fungal biomass, growth rate and lag phase, *P. nameko* and *P. ostreatusroseus* strains were the fungi that best tolerated LiCl.

Interestingly, LiCl affected the hyphae diameter and the septa distance differently (Figures 4, 5, 1S and 2S). *P. nameko* (PH 01; $P < 0.001$), *Grifolla* sp. (GF; $P = 0.019$), *H. herinaceum* (HE 01; $P = 0.004$), *G. subamboinense* var. *laevisporum* (GR 117; $P = 0.002$) and *P. eryngii* (PLE 04; $P = 0.001$) showed changes in the septa distance and *P. nameko* (PH 01; $P = 0.004$), *G. frondosa* (GF-JP; $P < 0.001$), *P. ostreatusroseus* (PLO 13; $P < 0.001$), *P. citrinopiliatus* (PLO A; $P = 0.004$) and *P. eryngii* (PLE 04; $P < 0.001$) altered their hyphae diameter when LiCl was added to the media culture. Though *L. edodes* (UFV 73; Septa distance $P = 0.112$; hyphae diameter $P = 0.506$) and *L. shimeji* (LY 01; septa distance $P = 0.566$; hyphae diameter $P = 0.108$) did not have altered septa distance or hyphae diameter, *L. edodes* and *L. shimeji* were sensitive to LiCl (Figures 2 and 3).

P. nameko (PH 01), *H. herinaceum* (HE 01), *P. ostreatus*(PLO 06), *L. shimeji* (LY 01), *G. subamboinense* var. *laevisporum* (GR 117), *P. citrinopiliatus* (PLO A) and *P. eryngii* (PLE 04) altered their macroscopic morphology when LiCl was added to the culture media (Figure 3S and Table 1). Additionally, the macroscopic morphology alterations varied among the fungi. A decrease in mycelial density was observed in four fungi (Table 1). This data, plus the dry mass data, clearly show that many fungi tested are sensitive to LiCl. *P. nameko* (PH 01) showed the highest decrease in mycelium density, and it was very difficult to see the hyphae fungus in the Petri dish at 1.65 g L^{-1} of LiCl (Figure 3S). A change in colony color was observed in three of the fungi tested, namely, *P. nameko* (PH 01), *G. subamboinense* var. laevisporum (GR117) and *P. citrinopilatus* (PLO A) (Table 1 and Figure 3S). *P. ostreatus* (PLO 06) and *P. eryngii* (PLE 04) were the only fungi that changed the color of the medium. This may suggest that these species secrete some compound in the presence of LiCl, even at the lowest LiCl level tested.

DISCUSSION

The increase in the lag phase is common when microorganisms are exposed to adverse environmental conditions (Swinnen et al., 2004; Yates and Smotzer, 2007). In this study, the addition of LiCl to the culture media increased the lag phase and decreased the growth rate of most of the fungi tested (Figures 1 and 2), showing that LiCl, at certain concentrations, can be toxic for the fungi. This information is important for production of Li-enriched mushrooms, because colonization of the substrate by the fungus is one of the key steps of mushrooms production. A high growth rate allows fast substrate colonization, reducing the possibility of contaminant growth. As observed by Richter et al. (2008), some common contaminants such as, *Aspergillus niger* and *Trichoderma* sp. are resistant to higher LiCl levels than those used in our experiments. Additionally, quick colonization reduces the incubation time and, reduces the time needed to produce mushrooms.

Knowing which species of white rot fungi are more sensitive to LiCl, it is important when selecting fungi tolerant to LiCl. Here we sorted the fungi in four groups (Table 2). Most fungi tested were sensitive to LiCl. Indeed, when compared with other fungi groups (Richter et al., 2008), basidiomycetes seems to be a group, in general, more sensitive to LiCl. The genus *Pleurotus* seems to be a group slightly more tolerant to LiCl (Table 2). Richter et al. (2008) showed that the *P. ostreatus* strain (MAD 542) was able to grow in 1.5 g L^{-1} of LiCl. However, those authors used media containing 20 g malt, while in this study, we used PDA media, which can influence the LiCl toxicity. Also, the addition of 0.5 g kg^{-1} of LiCl to coffee husks, it does not alter mushroom formation of *P. ostreatus* (de Assunção et al., 2012).

The most tolerant fungi are *P. nameko* (PH 01) and *P. ostreatusroseus* (PLO 13). *P. nameko* did not change its growth rate at any LiCl level tested, although this fungus decreased its dry mass by 70.73% at 1.20 g L^{-1} LiCl

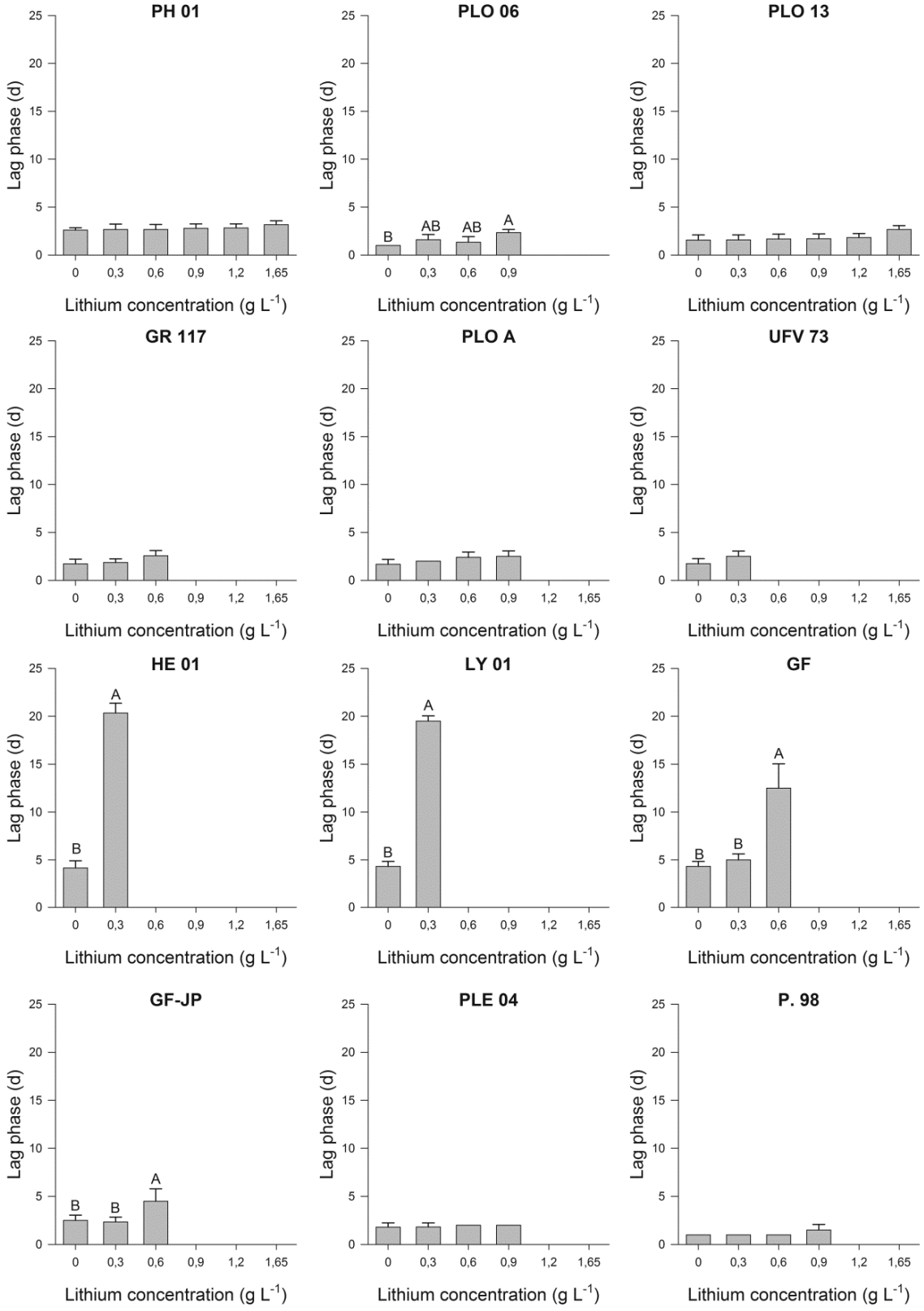

Figure 1. Lag phase of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicate fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

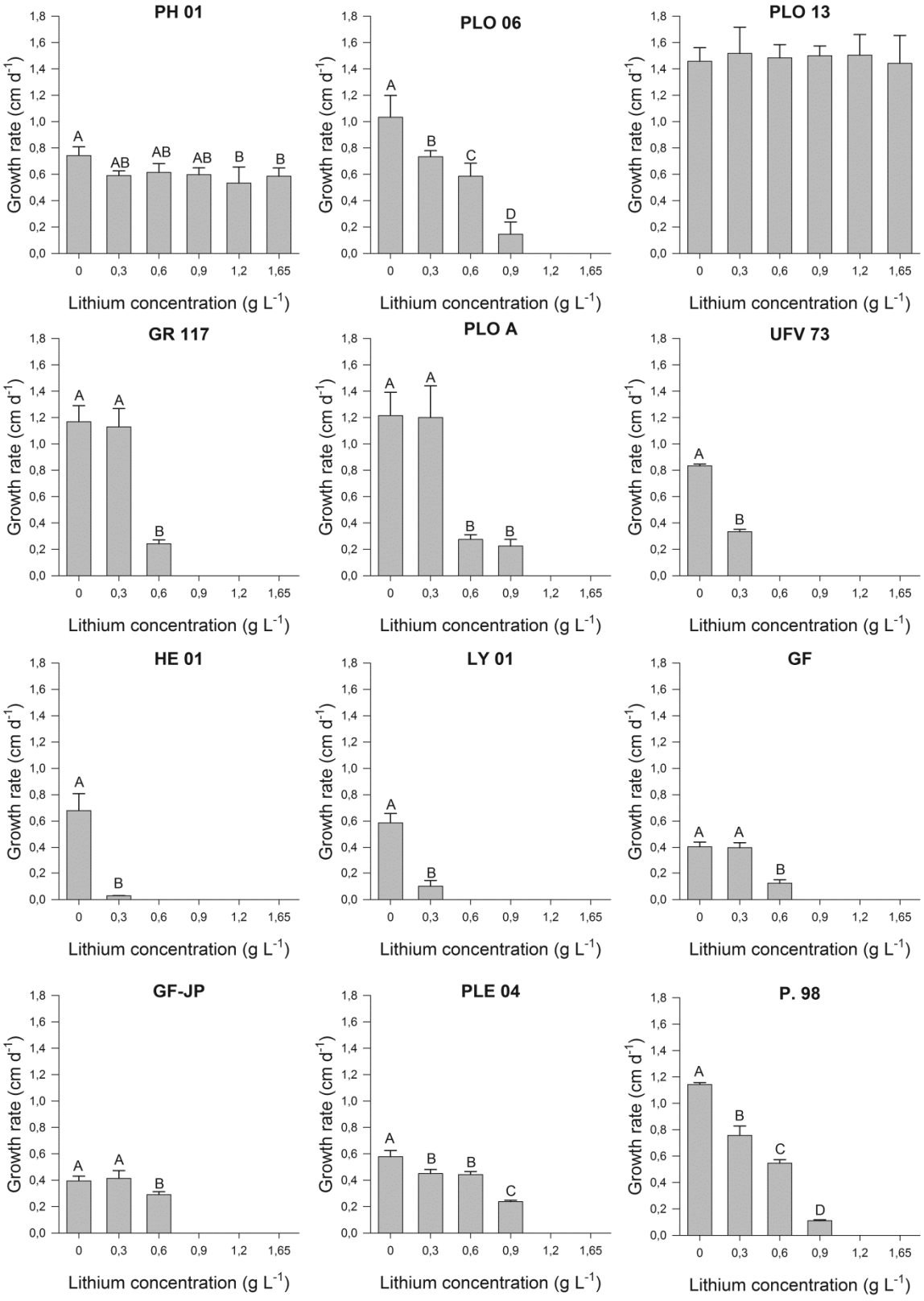

Figure 2. Mycelial growth rate of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicates fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

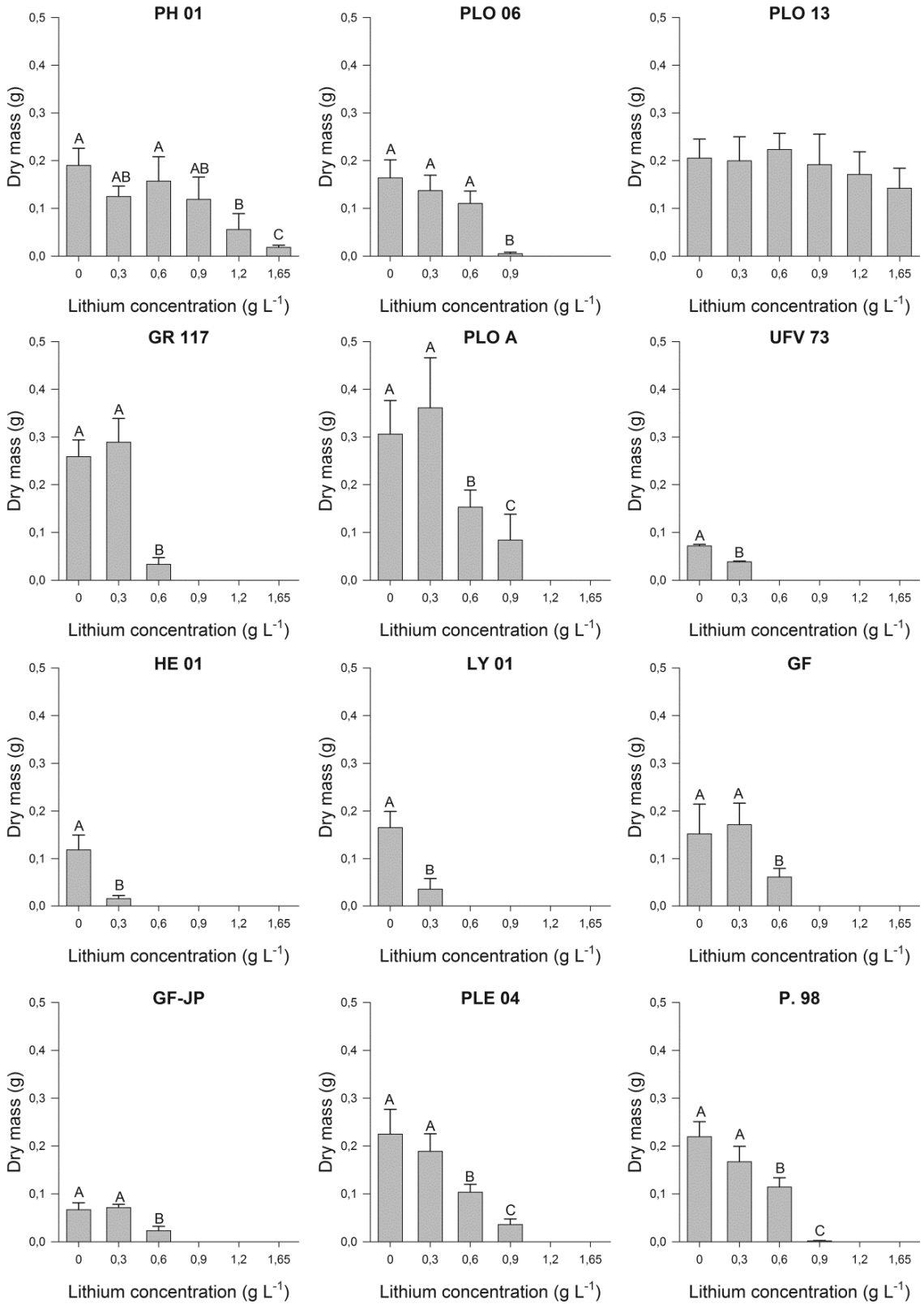

Figure 3. Mycelial dry mass of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicates fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

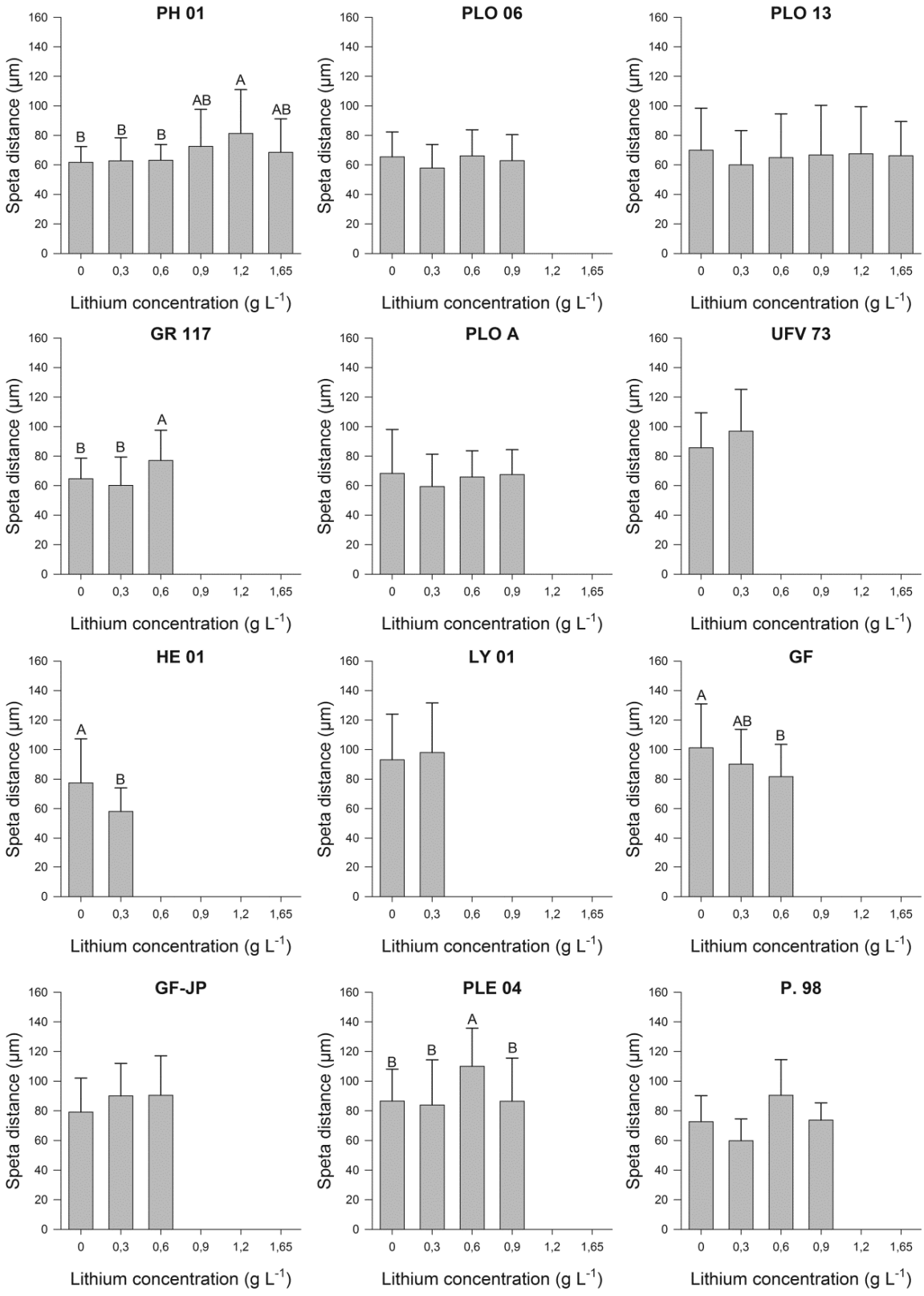

Figure 4. Septa distance of hyphae of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicate fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

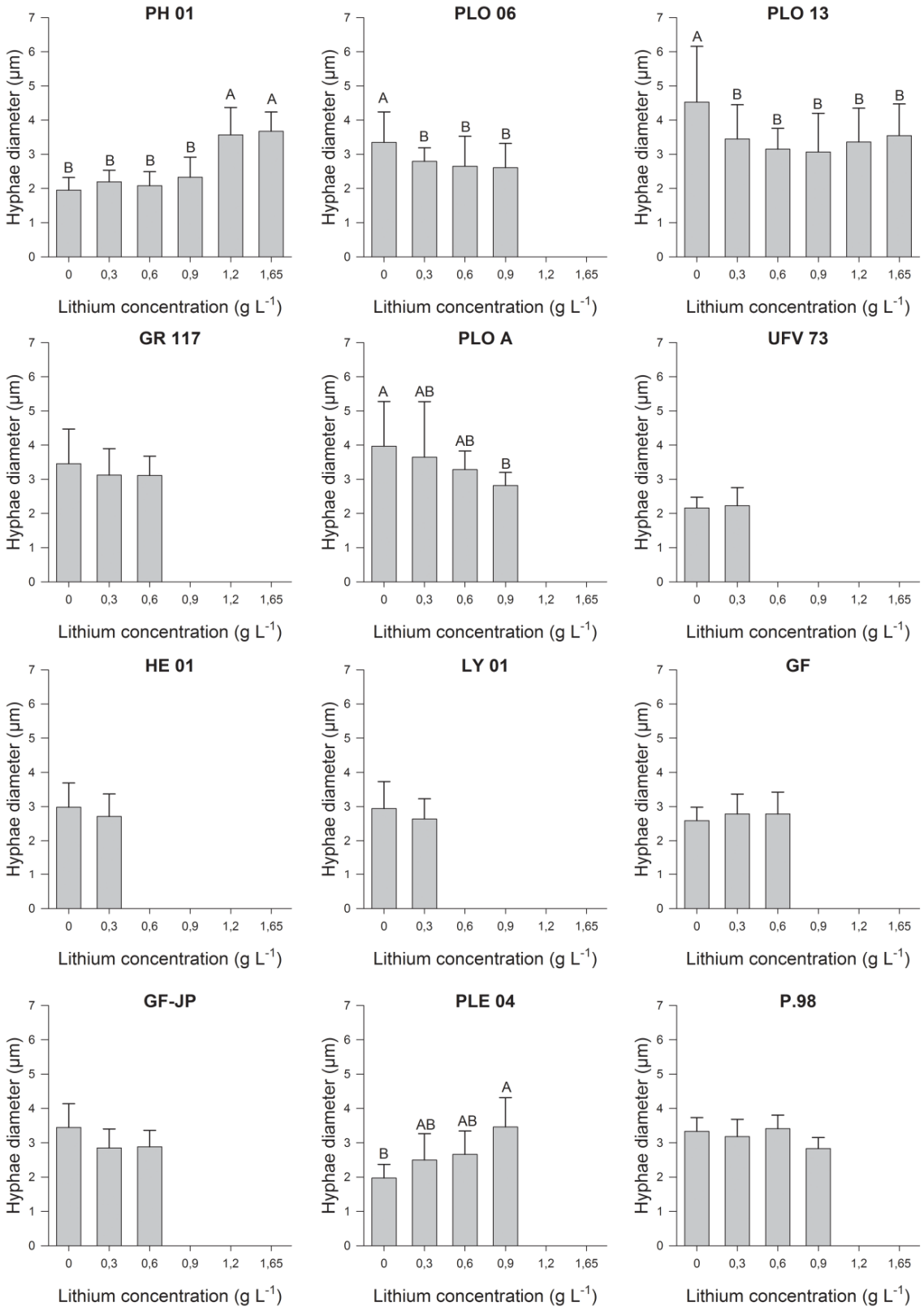

Figure 5. Hyphae diameter of white rot fungi in culture media supplemented with LiCl. Mean growth of 6 plates. Missing bars indicate fungal growth inhibition. Letters above columns compare individual fungi by LiCl level (Tukey´s test; $p \leq 0.05$).

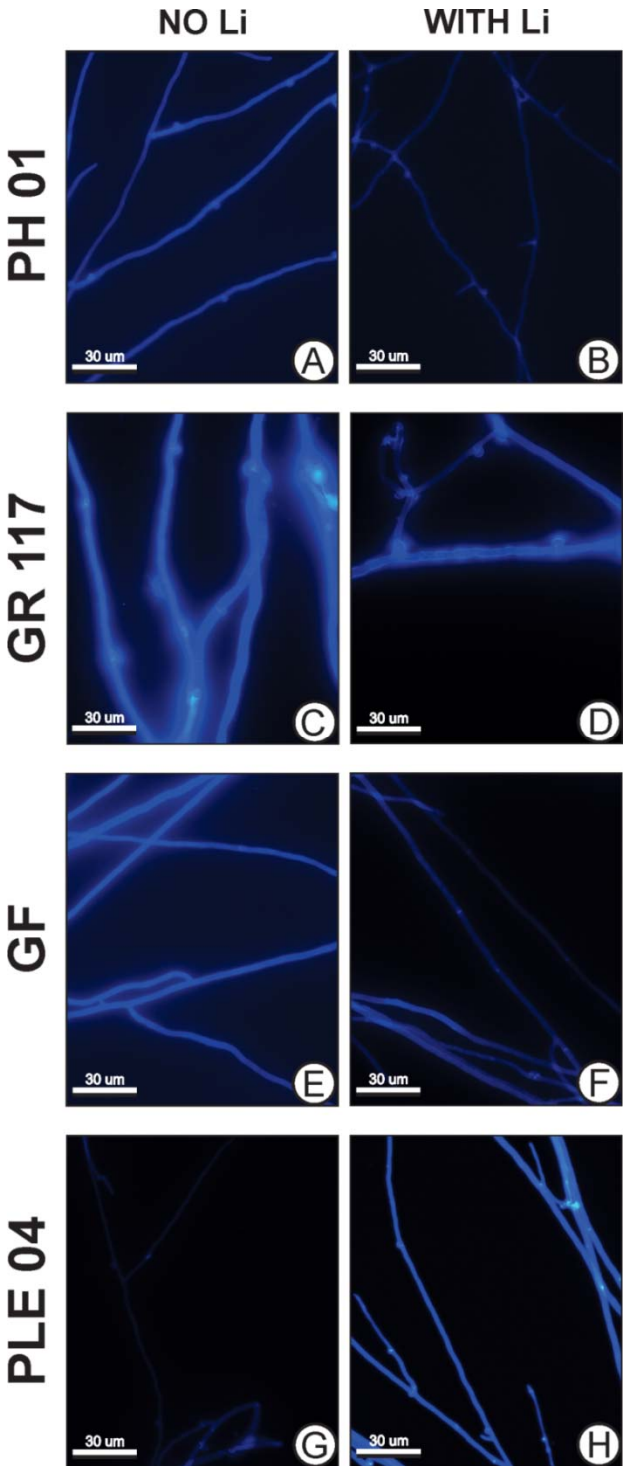

Figure 1S. Septa distance of white rot fungi in culture media supplemented with LiCl.

(Figure 3). This behavior suggests that *P. nameko* directs much of it energy to radial mycelial growth to look for environments with more suitable conditions. *P. ostreatusroseus* tolerated all the LiCl levels tested. Only the hyphae diameter decreased in the presence of LiCl (Figure 4), but the growth rate (Figure 2) and dry mass (Figure 3) did not change. As Li absorption by mushrooms can be linear and is directly related to the concentration of Li available in the substrate (de Assunção et al., 2012), further mycelial and mushroom production studies at higher LiCl level than we tested should be performed with this fungus, because production of mushrooms with high Li concentration can be interesting.

The morphological, macroscopic and microscopic changes were evaluated to begin to understand the response of fungi to LiCl. The modification of colony color suggests that some fungi are producing some compounds which are able to growth in the presence of LiCl. This hypothesis is further supported by the media color modification that occurred during the growth of *P. ostreatus* (PLO 06) and *P. eryngii* (PLE 04) strains (Table 1 and Figure S3). Further metabolomic studies should be performed to identify the intracellular or extracellular compounds responsible for the changes in color.

Some fungi increased their septa distance and hyphae diameter, while others decreased them (Figures 4, 5, 1S and 2S), showing that the fungi uses different adaptive strategies in environments with LiCl. Different taxonomic groups of fungi have previously been shown to decrease their septa distance under stressful conditions (Thrane et al., 1999; Turner and Harris, 1999; McIntyre et al., 2001; Denisov et al., 2011). The septum compartmentalizes the hypha without harming communication among them due to the presence of pores in the septum. (van Driel et al., 2008). When the cell is damage, the fungus may block the septa pores as a way to protect the colony (van Driel et al., 2008). Thus, colony with more septa will be more protected. We suggested that this strategy could be used by some fungi in environments with LiCl. Those fungi strains may increase the number of septa to reduce the time required to stock any damage that the colony may suffer due to the presence of the LiCl. Moreover, this strategy could also be used to reduce the portion of the colony that may suffer some damage.

Alteration in hyphae diameter is another morphological modification made by fungi (McIntyre et al., 2001; Tripathi et al., 2009; Dávila Costa et al., 2011; Denisov et al., 2011). Decreased hyphae diameter may decrease the energy required to produce a cell. The fungi could use this energy to look for a more suitable environment (e.g. redirect this energy for mycelial growth). On the other hand, increased hyphae diameter could be related to increased cell wall thickness. Chitin synthesis has been shown to increase when filamentous fungi are exposed to Calcofluor White (Ram et al., 2004). Additionally, yeast increases the chitin in its cell wall in response to stress (Lagorce et al., 2002). This increase in chitin content may be due to a higher chitin level in the lateral walls.

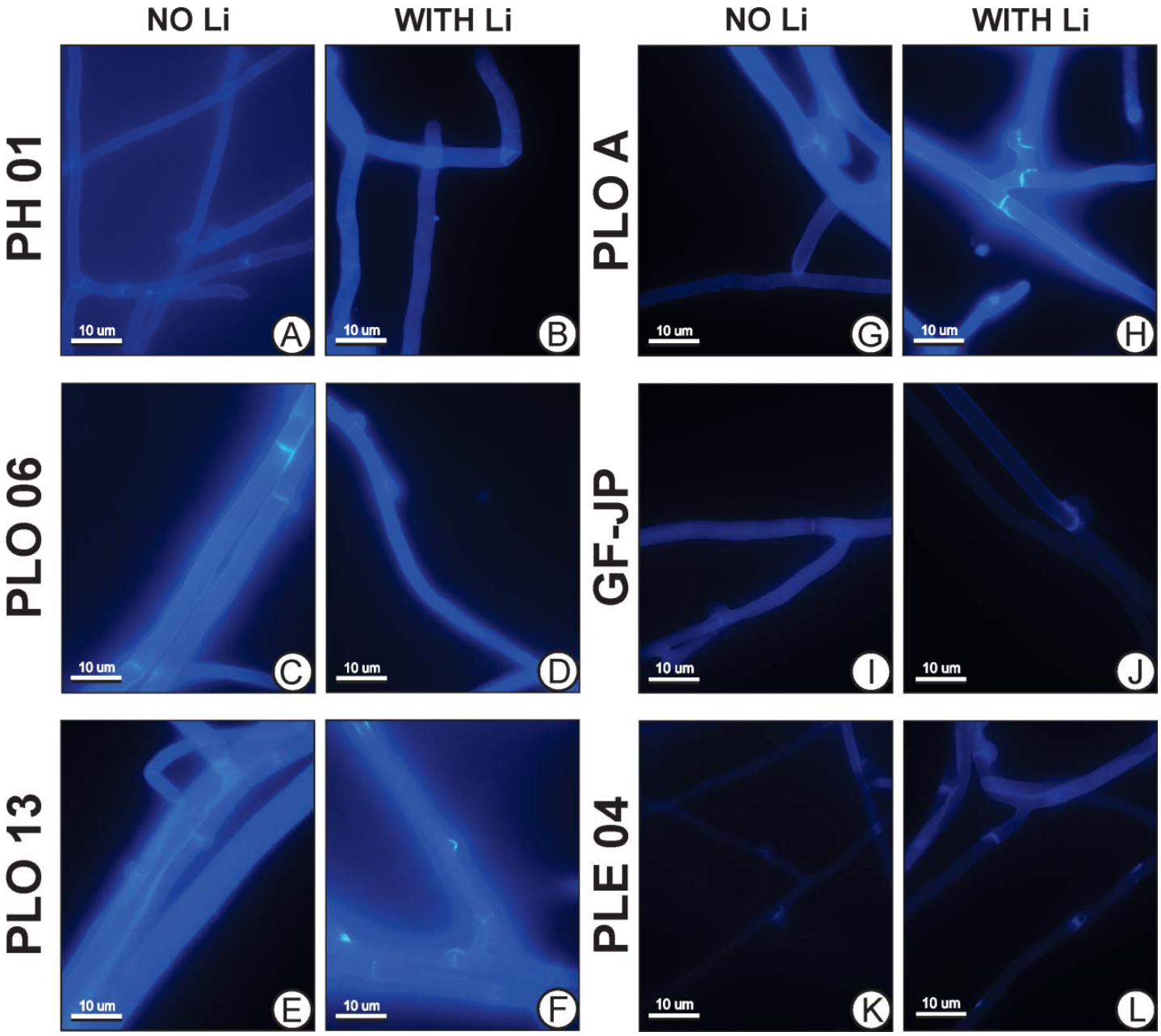

Figure 2S. Hyphae diameter of white rot fungi in culture media supplemented with LiCl.

Therefore, some white-rot fungi may protect the cells increasing the chitin content of those. This could be a strategy to avoid or reduce the entrance of Li into the cell. Increased hyphae diameter may also be related to cell swelling as observed by Lanfranco et al. (2002) where an ericoid ascomycete was treated with Zn. Indeed, the entrance of Li into the cell increases the osmolality of the cell, inducing the entrance of water. This consequently will result in cell swelling. The elucidation of these modifications could help to understand the process of fungal tolerance of LiCl and may help develop Li-enrichment methodology.

This is a preliminary and screening study. Other edible fungi may be more tolerant to LiCl, and thus more appropriate for Li-enrichment. Indeed, Richter et al. (2008) showed that *Trametes versiculor*, a fungus usually used in Chinese medicine, grows well in LiCl concentration up to 1.5 g L^{-1}. The aim of our research line is to produce Li-enriched mushroom. From this starting point, further studies should be performed. These include

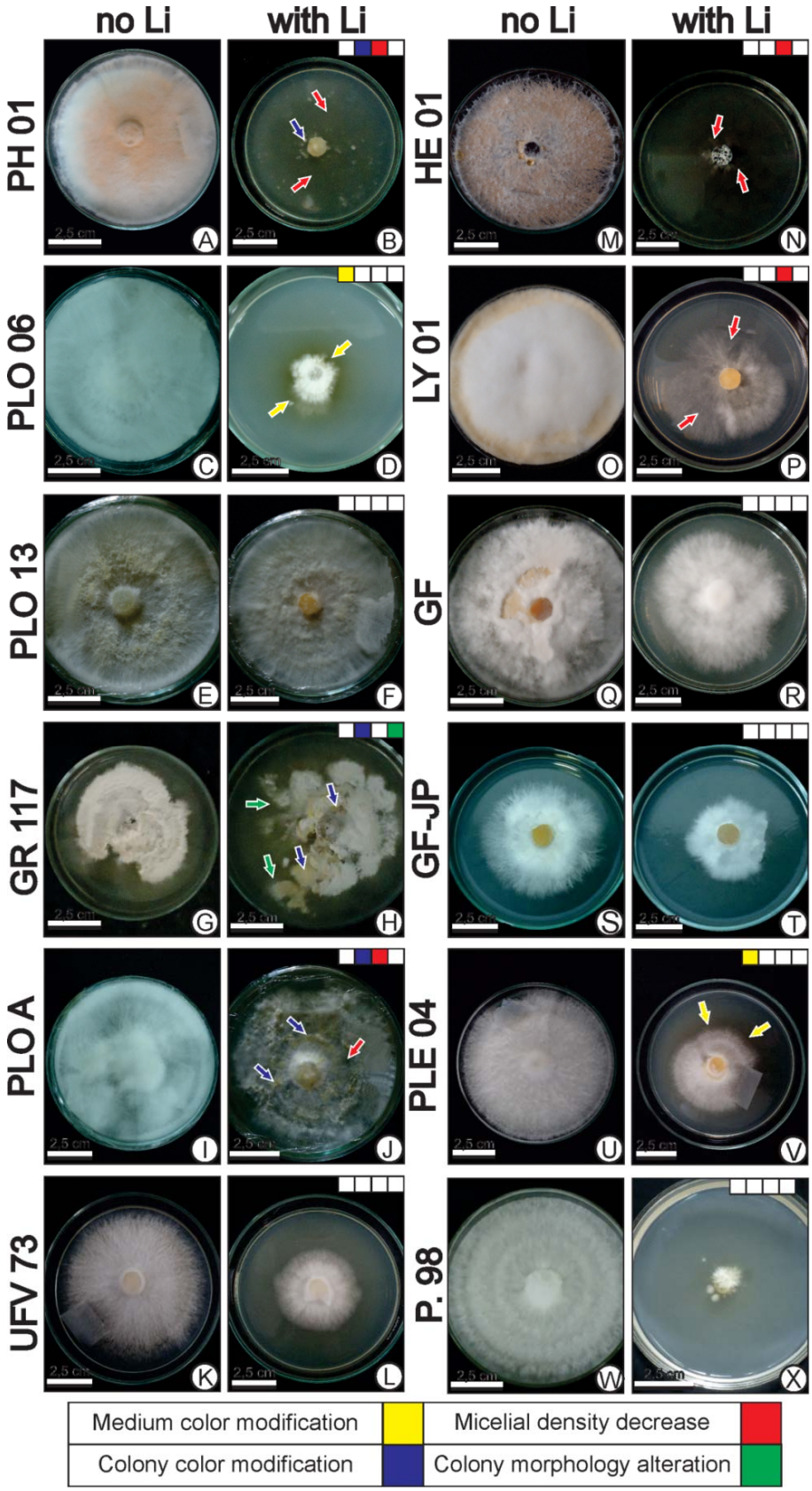

Figure 3S. Mycelial morphologyof white rot fungi in culture media supplemented with LiCl.

Table 1. Fungal macroscopic morphological alterations observed due to the addition of lithium chloride (LiCl) in the culture media.

Strain	Medium color alteration	Colony color alteration	Mycelial density decrease	Colony morphology alteration
PH 01		The mycelium light brown color that is intrinsic to this strain began to disappear with the increasing of LiCl	The mycelium thickness decrease with the increasing of LiCl and above 1.2 g L^{-1} the mycelium become so thin that was difficult to visualize the same in the Petri dish.	
PLO 06	Up to 0.3 g L^{-1} of LiCl we observed appearance of light orange color in the medium below the mycelium. The intensity of the orange do not changed with the increasing of Li concentration			
PLO 13				
GR 117		In higher concentrations of LiClwe observed the appearance of mycelium with light brown color.		In higher concentrations of LiCl we observed that the colony growth was not uniform.
PLO A		With the increase in LiCl concentration we observed an increase in the appearance of light brown mycelium.	Mycelium thickness decrease from 0.9 g L^{-1} of LiCl	
UFV 73				
HE 01			We observed thin mycelium at the lowest concentration of LiCl tested.	
LY 01			We observed thin mycelium at the lowest concentration of Li tested.	
GF				
GF-JP				
P. 98				
PLE 04	Up to 0.3 g L^{-1} of LiCl we observed appearance of light orange color in the medium below the mycelium. The intensity of the orange do not changed with the increasing of Li concentration.			

evaluation of mushroom production, measurement of the bioaccumulation of Li at different LiCl levels, the use of other sources of lithium and the effect of other substrate composition in the accumulation of Li.

Conclusions

Most of the Basidiomycetes strains used here showed sensitivity to LiCl. *P. ostreatusroseus* (PLO 13) was the only strain that was tolerant to all LiCl level tested, making it a promising fungus for future Li-enrichment research.

The fungi tested presented different morphological changes in the presence of LiCl at different concentrations. Elucidation of these various adaptive strategies may lead to new approaches for Li-enrichment.

Conflict of Interests

The author(s) have not declared any conflict of interests.

Table 2. Ranking of fungi sensitive to LiCl.

Group
Intolerant (growth up to 0.3 g L^{-1})
Hericium herinaceum
Lyophillum shimeji
Lentinula edodes
Sensitive (growth up to 0.6 g L^{-1})
Grifollafrondosa (GF-JP)
Grifollafrondosa (GF)
Ganoderma subamboinense var. laevisporum
Moderately sensitive (growth up to 0.9 g L^{-1})
Pleurotus ostreatus (PLO 06)
Pleurotus ostreatus (P. 98)
Pleurotus eryngii
Pleurotus citrinopiliatus
Resistant (growth up to 1.65 g L^{-1})
Pholiota nameko
Pleurotus ostreatusroseus

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