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Physiological implications of trehalose in the ectomycorrhizal fungus *Pisolithus* sp. under thermal stress

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Abstract

The trehalose cycle in filamentous fungi has been suggested to be an important mechanism of tolerance against adverse stress conditions, particularly in thermal stress. Here, we demonstrate that trehalose and trehalase activity can be involved as an additional mechanism in development of thermotolerance in *Pisolithus* sp. In response to heat shock at 42 °C, an accumulation of intracellular trehalose and an increase in trehalase activity in the mycelial mass of the fungus was observed, when compared to the control treatment (28 °C). In vitro, assays showed that trehalose had a protective effect on β-glucosidase activity under thermal stress. Therefore, trehalose production may be an important mechanism of protection in ectomycorrhizal fungi, and this capacity could be used in the selection of isolates with greater capacity for adaptation to environmental stress.
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Keywords: Cellular protection; Ectomycorrhizal fungus; β-Glucosidase; Thermal stress; Trehalase

1. Introduction

Most organisms have developed tolerance mechanisms to escape adverse environmental conditions. One such mechanism is the synthesis and accumulation of protective organic compounds, like sucrose, glycogen, and trehalose (Thevelein, 1984; Argüelles, 2000; Fillinger et al., 2001). Indeed, trehalose has been reported as a part of the physiological adaptation to various abiotic stresses in yeasts and filamentous fungi (Plesofsky-vig and Brambl, 1993; Singer and Lindquist, 1998a, b; Fillinger et al., 2001; François and Parrou, 2001; Elbein et al., 2003).

Trehalose is found in a variety of eukaryotic and prokaryotic organisms (Elbein, 1974; Jorge et al., 1997; Singer and Lindquist, 1998a, b), including filamentous fungi belonging to basidiomycete (Gancedo and Flores, 2004; Bois et al., 2006). At first, trehalose was considered as an energy source for cells, but later studies demonstrated that trehalose was also important in other metabolic processes, mainly, when organisms are exposed under adverse growth conditions (Singer and Lindquist, 1998a, b; Fillinger et al., 2001). In *Saccharomyces cerevisiae*, the accumulation of trehalose was observed when the cells were exposed to thermal shock (Argüelles, 1997; Fillinger et al., 2001), and its synthesis was mediated by trehalose 6-phosphate (T6PS) synthase and trehalose-6-phosphate phosphatase (T6PP). The hydrolysis of trehalose occurs by the action of trehalases (acid trehalase and neutral trehalase) and by a trehalose phosphorylase (Jorge et al., 1997; Wannet et al., 1998; Parrou et al., 2005). Previous work has shown that trehalose concentration was positively associated with the viability of cells under supra-optimal temperatures and other stress conditions (Attfield, 1987; Fernández et al., 1998; Silljé et al., 1999; Fillinger et al., 2001).

Some studies have sought to determine the mechanism of cellular protection during stress conditions by trehalose, for instance; in stabilizing intracellular enzymes and preventing the aggregation of denatured proteins (Singer and Lindquist, 1998a, b; Fillinger et al., 2001; François and Parrou, 2001; Elbein et al., 2003). Trehalose acts also as a stabilizer of cellular membranes during stress conditions (Patist and Zoerb, 2005). In addition, it has been reported

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that trehalose acts as signal molecule in metabolism in some organisms studied, including filamentous fungi (Parrou et al., 1997, 2005; Fillinger et al., 2001).

In ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi, trehalose together with mannitol and arabitol represent the majority of carbohydrates found in mycorrhizal roots and in mycelium (Martin et al., 1984; Söderström et al., 1988; Ramstedt et al., 1989; Smith and Read, 1997; Bois et al., 2006). Mycorrhizal fungi are of great ecological importance due to mutualistic plant–fungus association. For example, ECM fungi play a major role in the biology and ecology of forest trees, affecting growth, water absorption, and protection against pathogens (Smith and Read, 1997). Plants with ectomycorrhizas are more tolerant to heavy metals, salinity and low water availability in soil. However, trehalose involvement and trehalase activity have been very little explored in these fungi under stress conditions (Martin et al., 1984; Smith and Read, 1997).

Trehalose and trehalase have been shown to be important as modulators of growth in filamentous fungi in adverse environmental injuries (Jorge et al., 1997; Fillinger et al., 2001; Parrou et al., 2005). In the present work, we reported an approach to determine the importance of trehalose and trehalase activity in an isolate of ECM fungus (*Pisolythus* sp. isolate RV82) under supra-optimal temperatures. Thus, the aims were to determine whether trehalose protected mycelial growth following exposure to supra-optimal temperatures. Furthermore, to determine whether supra-optimal temperatures affected trehalose content and trehalase activity in the mycelial mass. The thermal stability of β-glucocidase was studied in the presence and absence of trehalose as a model enzyme due to its major role in ectomycorrhizal fungi metabolism.

2. Materials and methods

2.1. Isolate and culture condition

Pisolithus sp. RV82, isolated from southeast region of Brazil in *Eucalyptus grandis* forests was analysed in the study. The isolate was cultured and maintained on a nutritional medium MNM (Marx, 1969) at 28 °C, and it is maintained as living cultures at Bioagro Institute (UFV).

2.2. Mycelial growth under supra-optimal temperature conditions

Mycelial discs (10 mm diameter) were removed with a cork borer from marginal growth of fresh MNM cultures and placed with mycelial side down in the center of plates (90 mm diameter) containing 20 ml medium MNM. The plates were incubated under grown optimal temperature (28 °C) of this fungus. After 7 days incubation, the plates were subjected to supra-optimal temperature conditions as previously described (Ferreira et al., 2005). After heat treatment, the diameter of the colonies was measured (each 24 h) in four positions on plate during 144 h.

2.3. Enzymatic extract

Mycelia of the isolate (discs of 10 mm diameter) were grown on 50 ml liquid medium MNM (Erlenmeyer of 125 ml) supplemented with malt extract (500 mg l^{-1}) and yeast extract (50 mg l^{-1}), and flasks were incubated at 28 °C. After 10 days incubation, mycelia were washed with distilled water and pressed among gauze layers to remove water excess. Soluble proteins were extracted with 4 ml of acetate buffer (40 mmol l^{-1} potassium acetate, pH 6.8, and 5 mmol l^{-1} β-mercaptoethanol), using a chilled mortar for homogenizing the mycelium on ice-cold conditions. Homogenates were centrifuged for 15 min at 20,000*g* (4 °C). Enzymatic extract of the supernatant was used to evaluate β-glucosidase and trehalase activities.

2.4. Stability of β-glucosidase under supra-optimal temperatures

β-Glucosidase activity in vitro was determined by hydrolysis of the substrate pNPG (4-nitrophenyl-β-D-glucopyranoside), resulting in the release of *p*-nitrophenol (pNPh) measured spectrophotometrically at 400 nm. The enzymatic assays were performed in Eppendorfs (1.5 ml) with a final volume of 900 µl, containing 750 µl of acetate buffer (40 mmol l^{-1} potassium acetate, pH 6.8), 50 µl of enzymatic extract and 100 µl of pNPG (25 mmol l^{-1} in acetate buffer). The activity of enzyme was evaluated under optimal (28 °C) or three supra-optimal temperatures (42, 46 or 48 °C) by a time of incubation from 0 to 15 min. After the heat treatments, the mixtures of reaction were incubated at 37 °C for 40 min and then heated for 3 min in a water bath (100 °C) to stop the reaction. After centrifugation at 12,000*g* for 5 min, absorbance of the supernatant was measured. A standard curve was prepared with known amounts of pNPh.

2.5. Effect of glucose and trehalose on β-glucosidase activity

β-Glucosidase activity was evaluated in vitro assays under sublethal temperature (46 °C) in presence of glucose or trehalose, with the concentrations of sugar ranging from 0 to 600 mmol l^{-1}. The assays were carried out in Eppendorfs with a final volume of 800 µl containing 200 µl of enzymatic extract and 600 µl of acetate buffer (25 mmol l^{-1} pNPG, pH 8.0) with or without of the sugars. The reactants were transferred to water both at 46 °C for 4 min and then cooled in an ice bath for 5 min. An aliquot of 200 µl of each sample was utilized to evaluate the amounts of pNPh released.