Mycelial growth of native strains of Neolentinus ponderosus and N. lepideus at different pH and their Pinus spp. Wood degradation

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Abstract

Neolentinus ponderosus and N. lepideus are two saprophytic fungi species used traditionally in Huehuetenango and Totonicapán, Guatemala. The degradative capacity of both species confers them potential for fruiting bodies production. This study evaluated the mycelial growth of two native strains of N. ponderosus and N. lepideus in malt extract agar (EMA) at different pH and the degradation of wood from two pine species in rot chambers during 12 months. pH 7.0 was the most appropriate for the mycelial growth of N. ponderosus and for N. lepideus were 5.0 and 5.6. The colonies of both strains showed fruity odor, velvety texture, regular to irregular edge, white color, with or without diffusible pigment, hyphae with 1-5 µm width, chlamydospores and clamp connections. Wood from Pinus tecunumanii and P. ayacahuite exhibit weight-loss percentages between 8.76 ± 5.58 and 12.07 ± 5.66, with N. ponderosus 145.2003 and N. lepideus 90.2002, respectively. In both cases reached the early stage of brown-rot decay. These results could be useful for future research that evaluate the fruiting bodies production in logs for food and commercial purposes.

Keywords: edible fungi culture, brown-rot decay, fruiting bodies, saprophytic fungi.

Introduction

The cultivation of edible mushrooms is a biotechnological process that contributes to recycle lignocellulosic agricultural and forest residues, since the mushrooms are consumed as food for humans and the degraded substrates can be used in several ways (Sánchez, 2004). There are 7,000 species of fungi that are considered edible in the world but only 200 have been studied for cultivation on an experimental basis and about ten have been produced on an industrial scale: Agaricus bisporus (JE Lange) Imbach, Auricularia spp., Flammulina Velutipes (Curtis) Singer, Grifola frondosa (Dicks.) Gray, Hypsizygus marmoreus (Peck) HIM. Bigelow, Lentinula edodes (Berk.) Pegler, Pholiota nameko (T. Itô) S. Ito and S. Imai, Pleurotus spp., Tremella fuciformis Berk. and Volvariella volvacea (Bull.) Singer (Chang & Miles, 2004).

New species of edible fungi have been successfully cultivated in recent years, Agrocybe cylindracea (DC.) Maire, Favolus tenuiculus P. Beauv., Hericium erinaceus (Bull.) Pers., Lepista nuda (Bull.) Cooke , Falo indusiatus Vent, Pleurotus albidus (Berk.) Pegler, P. citrinopileatus Singer and Stropharia rugosoannulata Farl. ex Murrill (Chang and Miles, 2004 are the ones that are outstanding ; Omarini, Lechner and Albertó, 2009; Lechner and Albertó, 2011; Bran, Cáceres, Gurriarán, Morales and Flores, 2014). For this reason that currently many studies have focused on the search for new species of wild edible fungi, to study their cultivation and thus be able to expand the number of species available for human consumption (Omarini et al., 2009).

In Guatemala there is a great variety of edible fungi and the use of 83 species that are consumed in 48 towns of 20 departments of the country has currently been documented (Morales, Bran, & Cáceres, 2010). Within this great variety of species, N. ponderosus (Fr.) Redhead & Ginns and N. lepideus (OK Mill.) Redhead & Ginns stand out, which are very popular for their consumption and commercialization in the departments of Huehuetenango and Totonicapán (Bran , Morales, Cáceres, & Flores, 2003a, 2003b).

The natural habitat of the Neolentinus genus is the wood of Pinus spp, where it causes brown rot (Pegler, 1893; Redhead & Ginns, 1985). In Guatemala N. ponderosus has been found on wood of P. tecunumanii Eguiluz & Perry, while N. lepideus develops on P. ayacahuite Ehren., Therefore, it is considered that both species are saprobia in nature and with the possibility of being grown in forest waste (Bran et al., 2003a). Therefore, in 2007 several native strains of these species were isolated and studies on *in vitro* growth were also carried out with different culture media and different temperatures; as well as the production of inoculum and fruit bodies with different disinfection treatments, in order to achieve the production of fruit bodies on lignocellulosic waste generated in the country (Bran, Morales, Flores, Cáceres, & Blanco, 2007).

Because there are no studies about mycelial growth under different culture conditions, this study evaluated the behavior of two native strains (N. ponderosus and N. lepideus) in the EMA medium at different pH, as well as their activity. wood degradator of P. tecunumanii and P. ayacahuite, respectively, as a preliminary step to

establish if possible their cultivation and production of fruiting bodies on logs of pine wood and other synthetic substrates (forms that are used successfully in cultivation L. edodes), as well as the possible application of the disinfection technique of the substrates by immersion in alkaline water.

Materials and methods

Reactivation of the N. ponderosus and N. lepideus strains: Two strains that are deposited in the Saprobios and Mycorrhizal Fungi Cepario, of the Department of Microbiology, School of Biological Chemistry, were used; Faculty of Chemical Sciences and Pharmacy, of the University of San Carlos de Guatemala. The code and the origin of the strains are: Neolentinus lepideus, 90.2002 (Aldea Panquix, Totonicapán) and Neolentinus ponderosus, 145.2003 (Aldea Bulej, San Mateo Ixtatán, Huehuetenango). They were seeded in Petri dishes with EMA medium and incubated at 26 ° C for two weeks. Subsequently, they were re-seeded in the mentioned culture medium and incubated for two more weeks.

Diameter calculation of the colonies at different pH level: the procedure was carried out according to that recommended by Stamets (1993) and Mier, Toriello and Ulloa (2002): The EMA culture medium (Merck®) was prepared, the pH was adjusted at values of 5.0, 7.0, 9.0 and 12.0 with HCl or 10% KOH and sterilized in an autoclave for 15 min at 121 ° C and 1.0 Kg / cm2. 20 mL of each were served in sterile disposable polystyrene Petri dishes. In the same way, EMA medium was prepared without pH adjustment (5.6), which was used as a control.

Later, 10 boxes (repetitions) of each medium were inoculated with a 0.5 cm2 segment of mycelium from each of the strains. The inoculated boxes were sealed and incubated at 26 ° C for 21 days. The diameter reached by the colonies was measured and recorded in two perpendicular planes (x, y axes) from which the average diameter (cm) was obtained. With the data obtained, the Excel® program created a database that included the average diameter (cm) of the colonies of each of the strains in the media adjusted to different pH and the control medium.

Chambers preparation of the colonized wood: The procedure was carried out according to Johansen, (1949) and Pérez, Pinzón and Echenique, (1977): The formaldehyde, alcohol, acetic acid (FAA) fixing solution was prepared as follows: Ethanol 95 % (50 mL), acetic acid (5 mL), formaldehyde (10 mL), and distilled water (35 mL). The wooden blocks were saturated with FAA solution in a Kitasato that was vacuum-stripped for 30 min. The FAA was replaced with distilled water and they were vacuumed for 5 min, then washed with potable water for 30 min. Sections 20 µm wide were cut from the radial face of the blocks with a sliding microtome. The sections were stained with the Cartwright method of picro-aniline blue, which was carried out as follows: 1% aqueous Safranin (30 s), distilled water (30 s, twice), picro-aniline blue at steam (30 s), distilled water (30 s twice), 50%, 70%, 95% and 100% ethanol (30 s each), 100% ethanol (2 min, two changes) and xylene at 100 % (3 min, two changes). Finally they were placed in 100% xylene and they were placed on slides containing a small drop of Entellán® resin and then coverslips were placed on them, they were

pressed, labeled and left to dry. The sections were studied and the distribution of the hyphae in the wood cells was observed.

Analysis of results: The diameter of mycelial growth of each strain at the evaluated pH (5.0, 7.0, 9.0 and 12.0) and the control (5.6), was statistically analyzed by means of a one-way analysis of variance, with a subsequent test of multiple Tukey comparisons (α = .05) in the SPSS 19.0® program, to show statistically significant differences. The macroscopic and microscopic characteristics of the colonies obtained at the evaluated pH were analyzed through a cluster analysis with the PAST® statistical program, through the development of dendrograms constructed from Euclidean distances, to observe morphological similarities between the treatments. . For the degradation of wood, no statistical analysis was performed, but the percentage of weight loss and degradation by the fungus in the plant cells was described. Results: the greatest mycelial growth diameter of N. ponderosus strain 145.2003 was observed at pH 7.0, which presented 8.25 (± 0.13) cm after 21 days of incubation. However, this diameter did not show a significant difference (p> 0.05) with those obtained at pH 5.0 and pH 5.6, which corresponded to the control. The diameters observed at pH 9.0 and 12.0 were 6.81 (0.87) cm and 5.71 (± 0.43) cm, respectively, which presented a significant difference between each other (p = .001) and with respect to the other evaluated pH (p <.05). Likewise, a decrease in mycelial growth was observed in alkaline pHs (Chart 1).

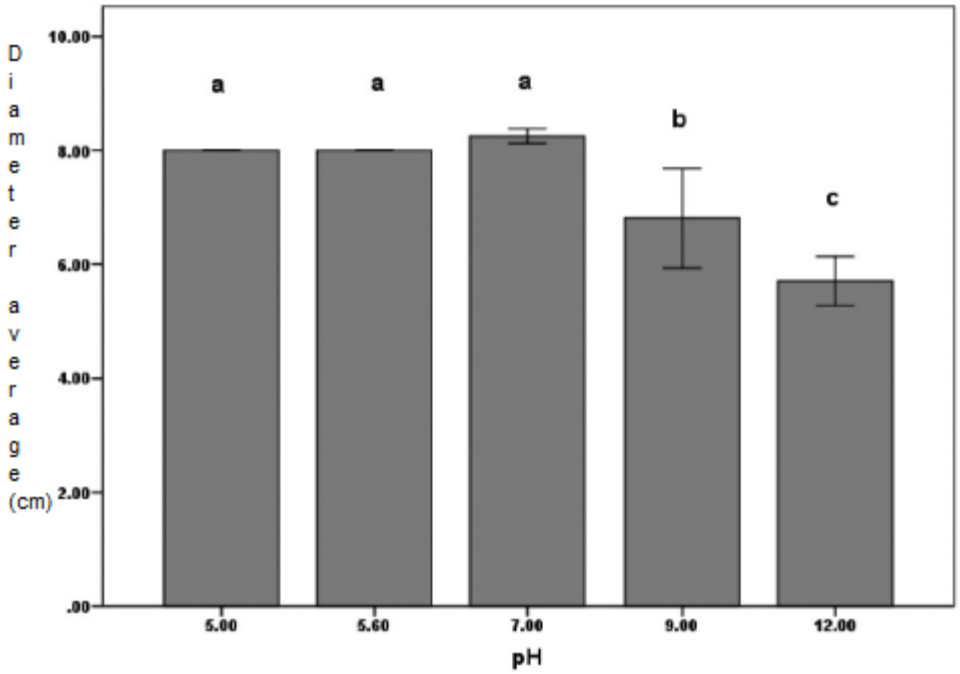

Chart 1. Mycelial growth diameters of N. ponderosus strain 145.2003 at five pH values, at 21 days of incubation. Bar graph indicates the mean of the mycelial growth diameter ± the standard deviation. Different letters indicate significant difference of Tukey's multiple comparisons test (p <.05).

The greatest growth diameter of N. lepideus strain 90.2002 was observed in pH 5.0, as well as in control pH (5.6), which presented an average of 8.0 (.00) cm in both cases after 21 days of incubation. Likewise, they did not show a significant difference between them (p> .05). The mycelial growth diameters observed at pH 7.0, 9.0, and 12.0, were 7.43 (0.13) cm, 4.94 (0.87) cm, and 4.26 (0.43) cm, respectively, showed significant difference between them (p <.05) and with regarding pH 5.0 and 5.6. A decrease in mycelial growth was also observed as the pH value increased (Chart 2).

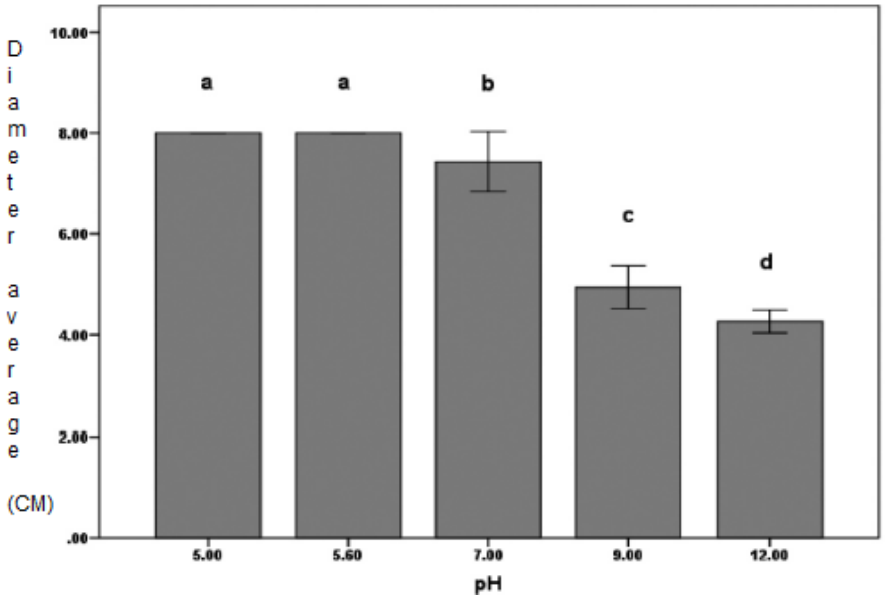

Chart 2. Diameters of mycelial growth of N. lepideus strain 90.2002 at five pHs, at 21 days of incubation. Error bars indicate the mean of the mycelial growth diameter ± the standard deviation. Different letters indicate significant difference with Tukey's multiple comparisons test (p <0.05).

Cluster analysis of N. ponderosus strain 145.2003 showed that the macroscopic characteristics of the colonies developed at the evaluated pH were initially associated in two groups (A and B) (Chart 3). Group A, which was divided into two subgroups (C and D). In subgroup C, the colonies obtained at pH 5.0 and 5.6 were grouped, which presented white colonies, absence of pigment, regular border, plush texture, sweet fruit odor, hyphae diameter of 1-5 µm (average 2.5 µm), presence of 2-3 fibulae per field at pH 5.0 and 3-4 fibulae per field at pH 5.6. In both cases, there were 0 to 1 chlamydospores per field. Subgroup D included colonies obtained at pH 12.0, which presented an irregular border, 1-2 fibulae and 2-3 chlamydospores per field.

Group B, which included colonies obtained at pH 7.0 and 9.0, showed white colonies, regular border, plush texture, sweet fruity odor, presence of diffusible pigment in the medium, yellowish to brown color in 60% of the colonies evaluated at pH 7.0 and in 100% of the colonies evaluated at pH 9.0, presence of 1-2 fibulae and 0-1 chlamydospores per field at pH 7.0, while 1-2 fibulae and 1-2 chlamydospores per field at pH 9.0.

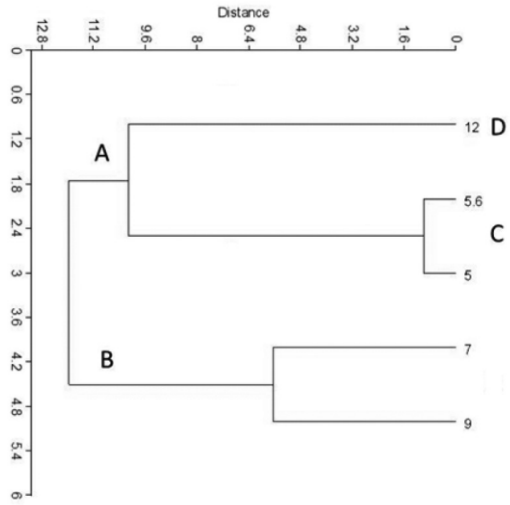

Chart 3. Analysis of clusters of macro and microscopic characteristics of N. ponderosus strain 145.2003 at five pH values.

Cluster analysis of N. lepideus strain 90.2002 showed that colony characteristics at the different pH levels evaluated were initially associated in two groups (A and B) (Figure 4). In group A included the colonies obtained at pH 7.0, which presented white coloration, regular border, plush texture, sweet fruity smell, presence of pigment, hyphae diameter of 1-4 µm (average 3.57 µm), presence of 3- 4 fibulae and 3-4 chlamydospores per field. Group B in turn was divided into 2 subgroups (C and D). Subgroup C included colonies developed at pH 12.0, which presented white coloration, plush texture, sweet fruit smell, presence of pigment, irregular border, hyphae diameter of 1-4 µm (average 1.73 µm), presence of 0-1 fibulae and 3-4 chlamydospores per field.

Subgroup D was divided into two more groups (E and F). Group E included the colonies evaluated at pH 9.0, which presented white coloration, regular border, plush texture, sweet fruit smell, hyphae diameter of 1-4 µm (average 1.85 µm), absence of pigment, 1-2 fibulae, and 3 -4 chlamydospores per field. Group F included colonies developed at pH 5.0 and 5.6, which showed white coloration, regular border, plush texture, sweet fruity odor, pigment in 20% of colonies evaluated at pH 5.0 and in 10% of those evaluated at pH 5.6, hyphae diameter of 1-4 µm (average 1.6 µm), presence of 3-4 fibulae and 0-1 chlamydospores per field.

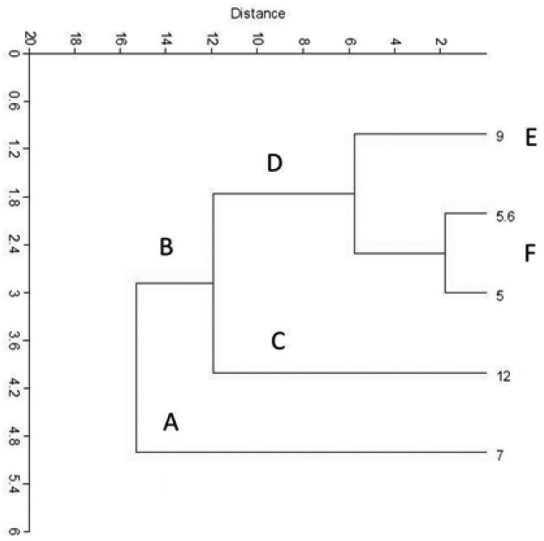

Chart 4. Analysis of clusters of macro and microscopic characteristics of N. lepideus strain 90.2002 at five pH values.

Regarding degradation, the weight loss of P. tecunumanii wood colonized with N. ponderosus strain 145.2003 was 8.76 (5.58)% (n = 29); while in the case of P. ayacahuite wood, when incubated with N. lepideus strain 90.2002, an average of 12.07 (5.66)% (n = 13) was obtained.

After 12 months of incubation of the wood in the presence of the two Neolentinus strains, it was observed that in both cases the brown rot caused only reached the early stage of degradation, since the hyphae only penetrated the wall until reaching the lumen of the cells.

Discussion

The concentration of hydrogen ions is very important since the state and availability of inorganic ions and the different metabolites in a culture medium depend on it. Most fungi can grow in an environment with a pH between 4 and 8.5, with an optimum between 5 and 7, very close to neutral pH (Cepero, Restrepo, Franco-Molano, Cárdenas, & Vargas, 2012).

In the case of the fungi N. ponderosus strain 145.2003 and N. lepideus strain 90.2002, it is very important to identify the optimal pH of mycelial growth, to subsequently improve the production of inoculum and fruit bodies. For this reason, since both N. ponderosus strain 145.2003 and N. lepideus strain 90.2002 obtained the highest mycelial growth in pH ranges 5.0-7.0, they are considered to be optimal for the growth of these strains. The reduction of mycelial growth in media with alkaline pH is due to the fact that some of the fungal genes are not expressed under these conditions, such as those that they are responsible for the expression of protease and oxidase enzymes (Polizeli & Rai, 2013).

Unfortunately, the reduction of mycelial growth of N. ponderosus and N. lepideus at alkaline pH would limit the use of the alkaline water immersion method to disinfect substrates in the production of fruiting bodies and may not be appropriate for the cultivation of these. mushrooms. However, it is

necessary to carry out other studies at the substrate level to verify the efficacy of this method in the cultivation of these two species.

When comparing the results obtained in other studies, it was found that they were similar to those obtained by Tandon (1961), who found that the fungi Phyllosticta cycadina, P. artocarpina, P. morfolia and P. mortoni showed better growth in the pH range. from 5.0 to 6.5. Likewise, they coincided with that reported by Berger and Hanson (1963), who observed optimal mycelial growth for Cercospora zebrina and Lophotrichus ampullus at pH 6.0. Currently, there are no studies evaluating the micellar growth of the N. ponderosus and N. lepideus species, therefore it is not possible to establish a comparison parameter.

On the other hand, the yellow pigment observed in the colonies in N. ponderosus strain 145.2003, at pH 7.0 and 9.0 and in N. lepideus strain 90.2002 mainly at pH 7.0, was probably due to the abundant production of secondary metabolites in these specific conditions and it did not affect the mycelial growth diameter of the colonies (Stamets, 1993).

Regarding wood degradation, using the Kumar and Dev classification, which assesses wood resistance based on weight loss, P. tecunumanii wood was classified as “very durable” as it lost between 0 and 10% of weight, while P. ayacahuite was classified as “durable” since it lost between 11 and 24% of weight. Both categories are considered with high resistance to fungal degradation (Kumar & Dev, 1993).

This was verified microscopically, since in both Pinus species, only the early stage of brown rot was evident, in which the hyphae reached only the lumen of the wood cells after penetrating the wall radially (Douglas, Flournoy, Kent, & Highley, 1991). For this reason, no changes were observed in the volume of the cell wall, nor the presence of cracks and / or indentations, characteristics of more advanced stages of degradation (Schwarze, Engels, & Mattheck, 2000).

These results demonstrated the high durability of the wood of these Pinus species, since despite the long period in which the wood remained in contact with the aforementioned strains, the cell structure was maintained, no evidence of advanced enzyme activity and high percentages of weight loss were found. These species could be used as substrates for the production of fruiting bodies, by means of the log cultivation method, as is done for other species such as Lentinula edodes (De León, 2010).

Similar studies were described by Ferraz, Rodríguez, Freer and Baeza (2001), who when incubating Pinus radiata wood with Wolfiporia cocos, a fungus that causes brown rot, for 90 days, obtained a weight loss percentage of 9.4 (0.4). Likewise, Chee, Farrell, Stewart and Hill (1998) incubated P. radiata wood with Artrodia serialis and Gloephyllum sepiarium, both brown rot fungi, for 126 days, and reported a weight loss percentage of 15.9 and 19.3, respectively. Although both publications show values comparable to that of the present study, they were obtained in a shorter incubation period, which may indicate that under the conditions of this study, the strains used degraded the pine wood evaluated more

slowly. Unfortunately, there are no similar studies for both fungal strains and Pinus species, so it is not possible to compare with the results obtained.

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It is important to mention that the study of the influence of pH on the growth of the native strains of N. ponderosus and N. lepideus, as well as the evaluation of wood degradation of the species of P. tecunumanii and P. ayacahuite, which are their natural hosts, constitutes a pioneering investigation in this field and determines the bases for subsequent studies, in which the production of fruiting bodies in trunks of these species of edible use is evaluated. in our country.

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