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Leucopaxillus giganteus Mycelium: Effect of Nitrogen Source on Organic Acids and Alkaloids

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The aim of this work was to find the most useful inorganic nitrogen source to enable *Leucopaxillus giganteus* to become a more nutritious mushroom, regarding organic acid and phenolic composition and total alkaloids content. For these, the influence of NH_4NO_3,$NaNO_2$, KNO_3,, and $(NH_4)_2HPO_4$ on the organic acid production was determined by HPLC-UV and total alkaloid content was assessed by a spectrophotometric method, after precipitation by Dragendorff's reagent. The results showed that *L. giganteus* presented an organic acid profile composed of oxalic, *cis*-aconitic, citric, and fumaric acids, citric acid being the major one. The quantitative organic acid profile and total alkaloid content were affected by the nitrogen source and depended on the developmental stage of mycelium and nitrogen availability. Despite being present in all samples, no phenolic compound could be identified.

KEYWORDS: ***Leucopaxillus giganteus*; inorganic nitrogen sources; organic acids; alkaloids**

INTRODUCTION

Mushrooms have been used as food and food-flavoring material in soups and sauces for a long time because of their typical and subtle flavor. Recently, they became attractive by virtue of their nutritional value and pharmacological properties such as antitumor and immunomodulating activity (*1–3*).

They are considered to present significant amounts of proteins, minerals, and vitamins, namely ascorbic acid, B vitamins, and vitamin D_2, as well as high contents of dietary fiber. On the contrary, lipid levels are low, which makes mushrooms an ideal food product to consume (*4–7*).

Among them, wild edible mushrooms have been largely consumed worldwide, not only because of their nutritional properties but also because of their taste, odor, and texture. Some species are a potentially important food, with good prospects for industrial use (*8*). In general, they are consumed fresh, dried during the off-season, or canned.

Leucopaxillus giganteus (Sowerby) Singer, also known as *Agaricus giganteus* Sowerby, *Aspropaxillus giganteus* (Sowerby) Kühner & Maire, *Clitocybe gigantea* (Sowerby) Quél., or *Paxillus giganteus* (Sowerby) Fr (*9*)., is commonly called giant funnel-shaped. It is a wild edible mushroom among the common species growing in Trás-os-Montes (northeast Portugal), a region rich in species of considerable gastronomic relevance. Because of its large size, only one mushroom is enough to be consumed by several people.

It is a saprophytic and gregarious mushroom that lives often in rings, fields, and meadows at the edge of woodland, growing during summer or autumn for three weeks or less. It has a pleasant, mealy odor and a good, sweet taste.

Few studies on *L. giganteus* are available in the literature. Previous studies concerned the effect of *L. giganteus* on bacterial content and nutritive substances in the soil (*10*), the determination of its sterol composition (*11*), the correlation of its antioxidant activity with its phenolics, ascorbic acid, β-carotene, and lycopene contents (*12*), and the evaluation of its phenol production and antimicrobial and antioxidant properties in the presence of different nitrogen sources (*13*).

Many mushrooms can access nitrogen from organic nitrogen-containing compounds (such as amino acids, amides, peptides, and proteins) and, to a lesser extent, from inorganic sources, such as ammonium and nitrate (*14*). Nitrogen sources generally play a significant role in mushroom cell proliferation and metabolite biosynthesis. Wasser et al. (*15*) demonstrated that the type and concentration of the N source strongly influenced the growth of the mushroom cell, either enhancing or decreasing it. As far as we know, nothing has been reported regarding the effect of the N source on the organic acid, phenolic compound, and alkaloid composition of this species.

The fruiting bodies of mushrooms are collected only in certain periods of the year. *L. giganteus* grows during summer and autumn seasons, but mycelium production allows collection over the entire year.

Our aim was to study the influence of four inorganic N sources (NH_4NO_3, ammonium nitrate; $NaNO_2$, sodium nitrite; KNO_3, potassium nitrate; $(NH_4)_2HPO_4$, diammonium hydrogen phosphate) on the organic acid and phenolic profiles and on

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Table 1. Characterization and Organic Acid Content of *L. giganteus* Mycelium Samples (mg/kg, dry basis)[a]

sample	nitrogen source	growth time (days)	oxalic (t_R = 18.0 min)	*cis*- aconitic (t_R = 22.0 min)	citric (t_R= 26.5 min)	fumaric (t_R= 54.8 min)	total
1A	NH_4NO_3	15	nq	2.3 (0.0)	3365.4 (39.5)	21.1 (0.6)	3388.8
1B	NH_4NO_3	30	nq	5.2 (0.3)	2008.1 (50.3)	41.1 (2.1)	2054.4
1C	NH_4NO_3	45	nq	11.2 (0.5)	1945.4 (19.9)	31.1 (0.3)	1987.7
1D	NH_4NO_3	60	nq	nq	1673.1 (6.8)	31.3 (0.2)	1704.4
2A	$NaNO_2$	15	nq	nq	7735.5 (314.4)	35.2 (0.8)	7770.6
2B	$NaNO_2$	30	nq	nq	5076.4 (113.4)	60.0 (1.1)	5136.5
2C	$NaNO_2$	45	nq	nq	897.6 (43.8)	4.2 (0.3)	901.8
2D	$NaNO_2$	60	nq	5.0 (0.1)	1997.1 (33.7)	2.9 (0.0)	2004.9
3A	KNO_3	15	158.2 (0.0)	3.8 (0.4)	2603.2 (50.5)	57.8 (0.7)	2823.1
3B	KNO_3	30	nq	7.7 (0.1)	2338.2 (38.0)	38.8 (0.7)	2384.8
3C	KNO_3	45	nq	13.7 (0.3)	1953.1 (93.4)	52.3 (0.4)	2019.1
3D	KNO_3	60	nq	nq	1693.9 (132.6)	55.8 (1.3)	1749.6
4A	$(NH_4)_2HPO_4$	15	215.6 (2.2)	4.7 (0.6)	3104.3 (85.0)	48.5 (1.8)	3373.0
4B	$(NH_4)_2HPO_4$	30	207.2 (5.4)	5.5 (0.1)	3105.8 (90.6)	43.6 (1.1)	3362.0
4C	$(NH_4)_2HPO_4$	45	143.5 (3.6)	6.7 (0.2)	1763.7 (48.4)	44.9 (0.4)	1958.8
4D	$(NH_4)_2HPO_4$	60	173.8 (0.1)	9.6 (1.4)	2336.9 (5.7)	41.1 (1.0)	2561.5

[a] Results are expressed as mean (standard deviation) of three determinations. nq, not quantified.

total alkaloid content of *L. giganteus* mycelium produced *in vitro*, in order to find the nitrogen source that promotes the highest production of those compounds and use this with commercial purposes.

MATERIALS AND METHODS

Standards and Reagents. Oxalic, citric and fumaric acids, and boldine and bismuth (III) nitrate pentahydrate were purchased from Sigma (St. Louis, MO). *cis*-Aconitic acid was from Extrasynthèse (Genay, France). Methanol and nitric, hydrochloric, sulfuric, formic and glacial acetic acids were obtained from Merck (Darmstadt, Germany). Potassium iodide was from Pronalab (Lisboa, Portugal). Thiourea and sodium sulfide x-hydrate were purchased from Panreac Quimica Sau (Barcelona, Spain). The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA).

Mycelia Culture: Biological Material. Mycelia of *L. giganteus* (Sowerby) Singer were isolated from sporocarps collected under grassland in Bragança (Herbarium of Agrarian School-Instituto Politécnico Bragança) and inoculated on solid Melin−Norkans medium, pH 6.6 (NaCl 0.025 g/L; $(NH_4)_2HPO_4$ 0.25 g/L; KH_2PO_4 0.50 g/L; $FeCl_3$ 0.050 g/L; $CaCl_2$ 0.50 g/L; $MgSO_4 \cdot 7H_2O$ 0.15 g/L; thiamine 0.10 g/L; casamino acids 1.0 g/L; malt extract 10 g/L; glucose 10 g/L; agar 20 g/L), following the method of Brundrett et al. (*16*). The strain was maintained in the same medium at 25 °C in the dark and subcultured every month.

Effect of Nitrogen Source on the Chemical Composition of Mycelium Mushroom. For aseptic establishment of the assay, 10 hyphal plugs (5 mm diameter) of 1-week-old *L. giganteus* mycelia were transferred into flasks (700 mL) containing 250 mL of liquid Melin−Norkans medium with four different N sources (NH_4NO_3, $NaNO_2$, KNO_3, and $(NH_4)_2HPO_4$) at a concentration of 0.25 g/L. Inoculated flasks were shacked and maintained in the dark at 25 °C. After 15, 30, 45 and 60 days of growth, the mycelium was recovered from the liquid medium by filtration, washed with distilled water, and dried at 50 °C, over 24 h. Three replicate flasks of mycelium for each growth period were used (a total of 12 flasks per nitrogen source). Each sample (**Table 1**) represents the sum of three replicates for each N source.

Extraction of Organic Acids, Phenolic Compounds and Alkaloids. For the chemical characterization of mycelia mushrooms, ca. 0.60 g of powdered material (910 μm) was boiled in 30 mL of water during 30 min and then filtered over a Büchner funnel. The resulting extract was frozen (−20 °C) and then lyophilized in a Labconco 4.5 Freezone apparatus (Kansas City, MO). A yield of ca. 0.36 g, on average, was obtained. The lyophilized extracts were kept in a desiccator, in the dark. For organic acids analysis, the lyophilized extracts were redissolved in 0.02 M sulfuric acid. For phenolic compound determination, water was used to redissolve the lyophilized extracts.

Phenolic Compounds Screening Tests. To check for the presence of phenolic compounds, 0.25 g of lyophilized extract was redissolved in 2 mL of water, and 20% NaOH and 4.5% $FeCl_3$ were added to two aliquots of the resulting solution.

Instrumentation. The HPLC system consisted of a 306 pump Gilson (Villiers le Bel, France), with an ion exclusion column Nucleogel Ion 300 OA (300 × 7.7 mm) from Macherey-Nagel (Düren, Germany) in conjunction with a column eating device model 7981, from Jones Chromatography (Essex, U.K.) and a UV detector Holochrome (Gilson) (Villiers le Bel, France). The HPLC control software was Gilson 712, version 1.3 (Villiers le Bel, France). The spectrophotometer was a Heλios α model, from Unicam (Cambridge, U.K.).

HPLC Analysis of Organic Acids. The separation of the organic acids was carried out as previously reported (*17*), in an analytical HPLC system in conjunction with a column heating device set at 30 °C. Briefly, elution was carried out isocratically with sulfuric acid 0.02 M, at a solvent flow rate of 0.2 mL min^{-1}. The detection was performed at 214 nm. Organic acid quantification was achieved by the absorbance recorded in the chromatograms relative to external standards, and the peaks in the chromatograms were integrated using a default baseline construction technique.

Total Alkaloids. The total alkaloid content of the samples was assessed using a method previously described (*18*) with some modifications. Total alkaloids were determined by a spectrophotometric method, in which they were precipitated by Dragendorff's reagent followed by the formation of a yellow bismuth complex in nitric acid medium with thiourea.

Dragendorff Reagent (DR). A 0.8 g amount of bismuth nitrate was dissolved in 40 mL of distilled water and 10 mL of glacial acetic acid. The resulting solution was mixed with 20 mL of 40% potassium iodide.

Calibration Curve. A 18.39 mg amount of bismuth nitrate was dissolved in 5 mL of concentrated nitric acid and diluted to 100 mL with distilled water to obtain a bismuth nitrate stock solution, from which a dilution series was prepared (379.1, 284.3, 213.3, 160.0, 120.0, 90.0, 67.5, 50.6, and 38.0 μmol/L). A 5 mL amount of thiourea 3% was added to 1 mL of each solution. The absorbance value of the yellow complex formed was measured at 435 nm. The assay was performed in triplicate. The average regression equation was $y = 6.83 \times 10^{-4} x$.

Assay. A standard solution was prepared by dissolving 5.00 mg of boldine in 5 mL of warm distilled water. For mushroom extracts, ca. 0.15 g of each lyophilized material was treated in the same way. Five mL of boldine solution/sample extract was adjusted to pH 2−2.5 (with 0.01 M HCl), and 2 mL of DR was added to form an orange precipitate that was centrifuged at 5000 rpm for 15 min. Afterward, DR was added to the supernatant to check for complete precipitation. A 2 mL amount of 1% sodium sulfide was added to the residue to form a brownish black precipitate which was centrifuged at 5000 rpm for 15 min. Compelete precipitation was checked by further adding 1% sodium sulfide. The resulting residue was dissolved in 2 mL of nitric acid with warming and sonication and then made up to 10 mL with distilled water.

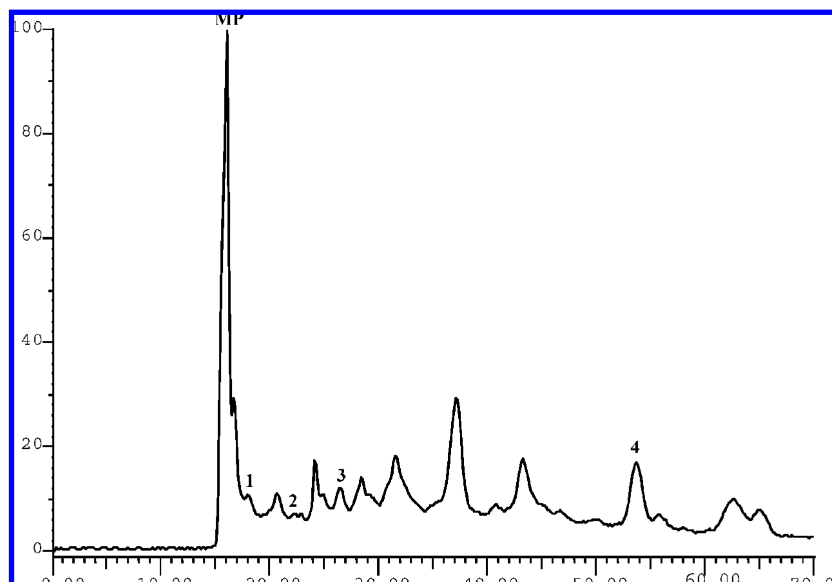

Figure 1. HPLC organic acid profile of *L. giganteus* mycelium with 15 day-old growth in a $(NH_4)_2HPO_4$ medium. Detection at 214 nm: (MP) mobile phase; (1) oxalic acid; (2) *cis*-aconitic acid; (3) citric acid; (4) fumaric acid.

A 5 mL amount of 3% thiourea was added to 1 mL of the resulting solution to form a yellow bismuth complex, of which the absorbance was measured at 435 nm. All the assays were performed in triplicate.

The amount of bismuth present in the boldine solution/extract was achieved from the calibration curve of bismuth nitrate. The results were expressed as boldine, considering that this is a monobasic alkaloid, and therefore the complex formed with bismuth follows a 1:1 stoichiometry.

RESULTS AND DISCUSSION

Organic Acids. *L. giganteus* mycelium growing under four different N sources showed a common qualitative profile composed by oxalic, *cis*-aconitic, citric, and fumaric acids (**Figure 1**). These compounds are reported for the first time in *L. giganteus* species. However, the organic acid quantitative profile was different for each N source, and it was not possible to clearly indicate the N substrate that enables the highest total organic acid production (**Table 1**). Citric acid was the major compound in all samples, ranging from 90.0 to 99.6% of all acids (**Figure 2**).

In cells, nitrate is converted into nitrite and then into ammonium ion. The intracellular ammonium ions are used to the amino acid production and, subsequently, to the formation of proteins, which have a marked influence on citrate productivity (*19*). Mushrooms have no cuticle to protect them from physical or microbial attack. The high water content makes them prone to microbial spoilage. This factor, in conjunction with age and enzymatic browning, causes textural changes in the mushroom. Citric acid has been used to extend the shelf life of mushrooms because of its antibacterial and antioxidant properties (*20*). The fact that this acid is present in *L. giganteus* samples in a high percentage emphasizes the importance of those properties in the analyzed mycelium mushroom. Citric acid has been known to be produced when the nitrogen source was the limiting condition (*21*). In our work, citric acid biosynthesis seems to be better achieved with NO_2, despite its relative amount is quite similar for the different N sources, with the exception of $(NH_4)_2HPO_4$ (**Figure 2**). These results are not in accordance with those from the literature, which report that the citric acid production is increased with the enhancement of ammonium concentration (*22*). In fact, the same work revealed that, under low concentrations of ammonium ion, the citric acid biosynthesis almost stopped, which led to the conclusion that ammonium ion controls citric acid biosynthesis. In order to enhance the antioxidant properties of the mushroom under study, any of the studied nitrogen sources could be used, especially $NaNO_2$, once all of them provide high citric acid levels.

Fumaric acid is an important organic compound because of its flavor, its antioxidant, antimicrobial, and acidifying properties (*23–25*). In the present work, fumaric acid amounts revealed big differences among the four different sources of nitrogen. In a general way, KNO_3 allowed the highest production of this acid, while $NaNO_2$ provided the lowest amounts (**Figure 2**, **Table 1**). This seems to indicate that KNO_3 could be useful to increase the fumaric levels in *L. giganteus* mushroom and, subsequently, provide the above referred properties, while the use of $NaNO_2$ would not be compensatory.

Regarding *cis*-aconitic acid, an increase of its levels is noticed during mycelium growth. However, for KNO_3 and NH_4NO_3, the rise of *cis*-aconitic acid amount only occurs through 45 days of development (**Table 1**). $NaNO_2$ would not be an advantageous N source for the production of high *cis*-aconitic acid levels.

The production of oxalic acid by *L. giganteus* mycelium mushroom was really influenced by nitrogen source. The N source that allowed the highest oxalic acid amounts was $(NH_4)_2HPO_4$. In all the remaining samples, it appears only in vestigial amounts, excepting for KNO_3, in which it was abundant in 15 day-old sample. Thus, in a general way, the accumulation of oxalic acid appears to be connected with the assimilation of ammonium (without nitrate) whereas nitrate (NH_4NO_3 and KNO_3) and nitrite ($NaNO_2$) forms seem exert inhibitory effects on oxalic production. This is not in accordance with previous works (*26, 27*), in which the results showed that oxalic acid increased as the ratio NO_3^-/NH_4^+ raises. Oxalic acid is found in living organisms as calcium salt, which is essentially insoluble in water. Calcium oxalate is the principal component of kidney stones and can be directly absorbed by the gut in spite of its insolubility. Although oxalic acid concentrations were low in the analyzed mycelium mushroom, its presence could be further reduced, or even prevented, applying the nitrate and nitrous substrates referred above. This could be important for human

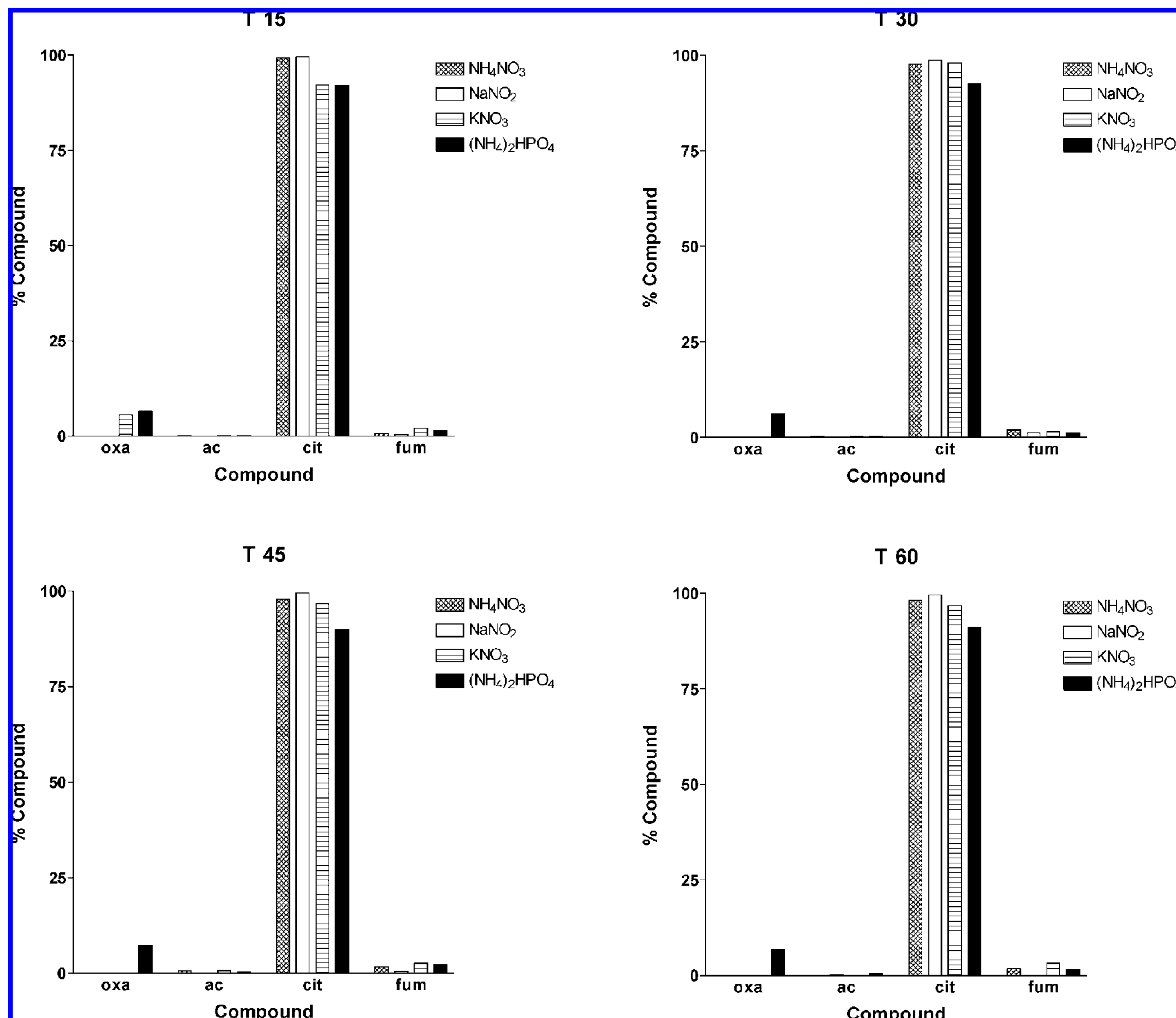

Figure 2. Organic acid profile of *L. giganteus* mycelium extracts developed in the presence of different nitrogen sources. T 15, T 30, T 45, and T 60 correspond to the days of mycelium growth. Organic acids: (oxa) oxalic acid; (aco) *cis*-aconitic acid; (cit) citric acid; (fum) fumaric acid.

health, especially for people with a history of calcium oxalate kidney stones.

By the analysis of **Table 1**, we can conclude that the organic acid production in *L. giganteus* mycelium decreases during its development for all the N sources, although for $NaNO_2$ and $(NH_4)_2HPO_4$, a rise has been observed after the 45th day of growth.

The results reported in a previous work (*13*) for the growth of the mycelium over time contrast with the data relative to the total organic acid production showed in **Table 1**. While the mycelium grown over 30 days, under the four different N sources, maintains its dry biomass after that, the total organic acid content decreased. In fact, in a general way, the mycelium growth phase corresponds to a decrease of the total organic acids content. This may occur because nitrogen sources are probably being used for organic acid production, which leads to a waste of nitrogen during the growth time, limiting the amounts of these primary metabolites in the mycelium mushroom.

Phenolic Compounds. The screening tests developed either with 20% soda or with $FeCl_3$ revealed the presence of phenolic compounds in all analyzed samples. The presence of these compounds in edible mushrooms is scarce (*28*). Despite the use of the same analytical conditions applied in previous work for the determination of phenols in other edible mushroom species (*17, 29, 30*), we have not managed to identify any phenolic compounds in *L. giganteus*.

Alkaloids. Alkaloids are secondary metabolites with basic character, containing heterocyclic nitrogen, and are synthesized from amino acids or their immediate derivatives (*31*).

It is known that alkaloid levels change depending on the nitrogen source used (*32–34*). In the present work, it seems that the sources containing nitrate (NH_4NO_3 and KNO_3) are not the ideal ones to promote the alkaloid synthesis by the mycelium (**Figure 3**). This is not in consonance with either previous works, indicating that nitrate increased the production of alkaloids more than ammonium sources (*32, 33*), nor with studies in which a combined source of nitrate and ammonium exerted a higher effect on the total alkaloid levels than the one achieved by only ammonium or only nitrate (*34*).

The maximum alkaloid production by *L. giganteus* mycelium, during development, in the presence of $(NH_4)_2HPO_4$ occurred at 60 days growth. However, for $NaNO_2$ and KNO_3 sources, the highest alkaloid production occurred at 15 days, while the

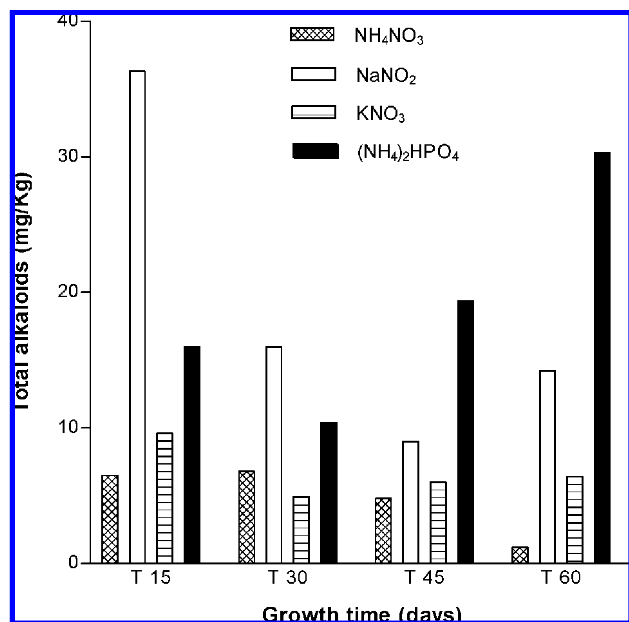

Figure 3. Total alkaloid content of *L. giganteus* mycelium extracts developed in the presence of different nitrogen sources. T 15, T 30, T 45, and T 60 correspond to the days of mycelium growth.

mycelium utilize nitrogen from NH_4NO_3 in a more efficient way through the 30th day (**Figure 3**).

As mentioned above, and according to previous work (*13*), mycelium grows abruptly in the initial days and then maintains biomass. On the contrary, in our samples the total alkaloid levels showed a tendency to decrease with age for mycelium supplied with $NaNO_2$ and NH_4NO_3 while those developed with $(NH_4)_2HPO_4$ exhibited a tendency to increase. The alkaloid production of the mycelium samples exposed to KNO_3 started from the first days of growth and runs in parallel to the biomass production. It seems that *L. giganteus* mycelium produces these secondary metabolites mostly in the initial days, which is in consonance with the results from the literature (*35*).

In conclusion, the results obtained in this study demonstrated that mushroom mycelium could constitute a good source of healthy compounds, namely organic acids and alkaloids. Nevertheless, we cannot ignore the possibility of the presence of toxic alkaloids, as observed in some mushroom species (*36, 37*). *L. giganteus* mycelium is especially rich in citric acid, which presents antibacterial and antiradical properties, being very important to prevent the browning of mushroom and to extend its shelf life. Thus, canned mycelium may not need high amounts of preservatives. Although the analyzed N sources do not show significant differences in citric acid production, $NaNO_2$ is the best choice to increase the levels of this acid. On the other hand, $NaNO_2$ and $(NH_4)_2HPO_4$ could be used to promote higher total alkaloid content. Further studies considering the determination of the effect of nitrite and nitrate on *L. giganteus* mycelium should be developed, regarding its possible commercialization with biotechnological or pharmaceutical purposes.

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