Gut passage of epigeous ectomycorrhizal fungi by two opportunistic mycophagous rodents

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Abstract  Mycophagists can influence fungal diversity within their home ranges by ensuring the continued and effective dispersal of spores from one site to another. However, the passage of spores through the digestive tract of vertebrates can affect the activity and viability of the spores ingested. This phenomenon has been rarely documented in opportunistic mycophagists consuming epigeous fungi. Using laboratory experiments, we investigated the activity and viability of spores of two epigeous ectomycorrhizal fungal species (Laccaria trichodermophora and Suillus tomentosus) after passage through the digestive tract of two opportunistic mycophagous small rodents, the volcano mouse Peromyscus alstoni and the deer mouse P. maniculatus. We found that passage through the gut of either species of rodent had a significant effect on spore activity and viability for both fungal species. The proportion of active spores (0.37–0.40) of L. trichodermophora in the feces of both species of rodents was less than that recorded for the control (0.82). However, the proportion of active spores (0.64–0.73) of S. tomentosus in the feces of each species of rodent was higher than in the control (0.40). On the other hand, the viability of spores was lower (0.26–0.30 in L. trichodermophora and 0.60–0.69 in S. tomentosus) for both fungi when consumed by either rodent relative to the controls (0.90 in L. trichodermophora and 0.82 in S. tomentosus). These findings suggest that these rodent species may be effective dispersers of both epigeous fungi [Current Zoology 57 (3): 293–299, 2011].

Keywords  Gut passage, Epigeous ectomycorrhizal fungi, Mexico, Spore activity and viability, Peromyscus

Numerous species of small mammals in temperate and tropical forests feed on sporocarps of mycorrhizal fungi (Maser et al., 1978; Polaco et al., 1982; Lamont et al., 1985; Blaschke and Baumler, 1989; Johnson, 1994; Claridge and May, 1994; Vernes et al., 2001; Maser et al., 2008). This eating habit is fundamental for the persistence of the mycorrhizal associations between fungi and trees and thus likely influences primary productivity of forests given that passage through the digestive tract of a mammal can enhance spore activity (metabolic activity) and may promote increased viability (germination capacity) (Trappe and Maser, 1977; Kotter and Farentinos, 1984; Cork and Kenagy, 1989; Frank et al., 2006, Maser et al., 2008). Beneficial plant-mycorrhizal interactions in the rhizosphere are primary determinants of plant health and soil fertility. Mycorrhizal associations are the most important microbial symbiosis for the majority of plants and, under conditions of phosphorus limitation, influence plant community development, nutrient uptake, water relations, and above-ground productivity (Jeffries et al., 2003). They also can act as bioprotectants against pathogens and toxic stresses (Azcón-Aguilar and Barea, 1996).

Mycophagous mammals can be grouped as obligate, preferential, or opportunistic according to the degree to which they depend on fungi in their diet throughout the year (Trappe and Maser, 1977; Johnson, 1996). Interestingly, most studies addressing the effects of small-mammal gut passage on fungal spore activity and germination have focussed on hypogeous fungal species (Johnson, 1996; Maser et al., 2008). By contrast, despite their diversity in most forest ecosystems, mycorrhizal epigeous fungi have received much less attention (but see
In this study, we focus on epigeous fungi, and we propose some hypotheses regarding the importance of small-mammal consumption and digestion to epigeous fungi ecology. By analyzing the presence of fungal spores in rodent feces of specimens captured throughout a 1-year period, Durán (2006) showed that volcano mice (Peromyscus alstoni Merriam) and deer mice (P. mani culatus Wagner) at La Malinche (Tlaxcala, Mexico) were important consumers of several species of mycorrhizal epigeous fungi. Results from this study indicated that spores of Russula, S. tomentosus and L. trichodermophora species are common in rodent feces, and that sporocarps of these fungi species are frequently consumed by rodents at the study site. In a subsequent study, D’Alva et al. (2007) showed that both of the previously mentioned rodent species feeding on Russula occidentalis (Singer), a fungal species whose spores are commonly found in the feces of these rodent species at La Malinche, had a minimal digestibility of energy and nitrogen (50%-60 %), suggesting that epigeous fungi are of moderate nutritional value to them. However, given that they represent an important source of water and an alternative resource when preferred food sources are scarce, and that both small rodent species consumed epigeous fungal species in large quantities at La Malinche, rodent mycophagy may play a relevant role in the dispersal of spores of these fungal species. Integrating these observations leads us to hypothesize that if spore viability is enhanced by digestion, then mycophagists have evolved tendencies to consume these fungi presumably for reasons other than nutrition, and possibly for reasons involving the maintenance of other woody-plant species that benefit them in other capacities (e.g., food, nest material, shelter). Alternatively, if the viability/activity of spores after gut passage is lower than controls post-digestion then there still may be a dispersal pay-off for the fungi that is worth the trade-off in reduced viability.

Thus, the goal of this study was to test experimentally whether passage through the digestive tracts of volcano and deer mice affects activity/viability of spores from epigeous ectomycorrhizal fungi Laccaria trichodermophora Mueller and Suillus tomentosus (Kauffman) Singer, Snell and Dick. Spore activity is related to mitochondrial respiration, which is indicative of metabolic activity (Torres, 1992), whereas spore viability is related to the presence of nucleic acids which may be indicative of the capacity of spore germination (Santiago-Martinez and Estrada-Torres, 19991; Montuega et al., 2009). Herein we use staining techniques to examine how digestion affects these two processes. We relate findings from this study to our knowledge of the ecological role of rodent species as dispersal agents of epigeous ectomycorrhizal fungi.

1 Santiago-Martínez MG, Estrada-Torres A, 1999. Hongos ectomicorrizógenos y producción de inoculantes para plantas de interés forestal. Fundación Produce Tlaxcala. México. Bulletin No. 19.

1. Materials and Methods

1.1 Study site

Specimens of both rodent species were captured, after formal approval of the relevant ethical local authorities (Secretaría de Medio Ambiente y Recursos Naturales-SEMARNAT) in September 2006 with medium-size Sherman traps at the Parque Nacional La Malinche, Tlaxcala, Mexico (19°14’ N, 98°58’ W; 2,900 meters above sea level). This locality presents a coniferous forest composed of Pinus montezumae Lamb., P. hartwegii Lindl., Abies religiosa (HBK) Schlecht et Cham., pastures and vegetation undergoing secondary succession. Mean annual precipitation is 800 mm, and the rainy season extends from June to October; mean annual temperature is 15°C.

1.2 Specimen capture and care

Rodents were kept in individual cages (40 × 20 × 20 cm³) at the laboratory in Tlaxcala city (Centro de Investigación en Ciencias Biológicas) under a 12:12 h light: dark photoperiod regime and ambient temperature (15°C). Specimens were maintained in captivity for a 3-week acclimation period, and all of them gained mass with rodent chow and water. The three males and three females captured of P. alstoni ranged from 21 to 41 g during the experimental period, and the four males and two females of P. mani culatus ranged from 16 to 21 g. All rodents used for the experiments were classified as adults based on identification field guides (Ceballos and Galindo, 1984), and they were released after the experiment at their capture site.

We focused on the two most common species of fungi observed in the diets of small-mammals in our study area (Durán, 2006). We chose the epigeous ectomycorrhizal fungi L. trichodermophora and S. tomentosus to assess the effects of rodent gut passage on the metabolic activity and viability (germination capacity) of spores because these two species of fungi are
frequently consumed by volcano and deer mice as well as other rodents at the study site (Durán, 2006). The sporocarps used in the feeding experiment were collected, characterized and taxonomically identified at the study site, in accordance with conventional techniques (Delgado et al., 2005).

1.3 Feeding experiments

Feeding trials were performed in September 2006. Using microscopic analysis, we verified that mice guts were clear of fungi spores. For seven consecutive days, the two experimental diets (L. trichodermophora or S. tomentosus) were randomly assigned to the rodents deprived of food for 8 h prior to the trials (three different animals of P. albonti and P. maniculatus for each diet). Specimens were housed in cages that permitted separate collection of feces and residual food. The animals received 60 g of mature sporocarps daily. Each day, feces were collected, characterized and taxonomically identified at the study site, in accordance with conventional techniques (Delgado et al., 2005).

1.4 Spore preparation and counts

Feces (55 g) collected throughout the study were transferred to 250-ml Erlen-Meyer flasks to which 30 ml of distilled water were added. To completely dissolve the feces, the solution was placed on magnetic stirrers for approximately 20 minutes then sieved twice, first with a 0.5 mm-diameter sieve and then with a 0.177 mm-diameter sieve, thus removing large pieces of waste materials. The sample was divided into test tubes (0.5 g in each). Samples were centrifuged for four minutes at 5,000 rpm. The supernatant was separated from the sediment, washed with 2 ml of distilled water and stored in stoppered glass bottles and were chilled to 4°C for subsequent spore counts.

To quantify the number of active and viable spores, we used two spore staining techniques, including staining with tetrazolium salts (MTT) which allowed us to determine mitochondrial activity in spores, and staining with hematoxylin to determine the presence of intact nuclei in potentially viable spores. Using the MTT technique, mitochondria of active spores are stained purple due to the release of succinate dehydrogenase which is one of the enzymes of the tricarboxylic acid cycle in fungi which is restricted to some places in the active mitochondria and reacts with tetrazolium salts (Miller et al., 1993). Similarly, tetrazolium salts form a red compound known as purple formazan when reduced by the mitochondria that contain nucleic acids that oxidize the hematoxylin to form a substance purple to dark purple called hematein (Montuenga et al., 2009). In the case of viable spores, nuclei are stained reddish or violet. Solutions of the feces were placed in a 1:3 ratio of glacial acetic acid/absolute alcohol remaining during three days, followed by centrifuging for five minutes at 2,500 rpm and removing the supernatant. Subsequently, we added 10 ml of 95% ethyl alcohol to the obtained solutions, stirred and centrifuged again, equating weights of all samples to 68.9 g. This process was repeated once more. The sample was then hydrolyzed with the addition of 3 ml of absolute alcohol and the following solution: 0.1 g AlNH4(SO4)2·12H2O, 0.1 g CrK(SO4)2·12H2O, 0.1 g iodid acid, and 3 ml of concentrated hydrochloric acid. After mixing, the sample was left to stand for 12 minutes to allow complete hydrolysis and this was followed by centrifugation for 5 minutes at 2,500 rpm. The supernatant was removed and the spores were transferred to a fixative containing absolute ethanol, chloroform and glacial acetic acid (6:3:1 respectively), followed by centrifugation. Finally, the spores were transferred to the stain containing 1.0 g of hematoxylin in 50 ml of propionic acid to 45% and 0.25gr Fe (NH4)2(SO4).12H2O. To assess the number of viable spores, the samples were analyzed using the same protocol as with MTT staining (Torres, 1992).

Sporocarp samples were used as controls. Both fungal species were blended with 400 ml of distilled water at medium high speed for 4 minutes and stored in stoppered glass bottles at 4°C. Previous studies have demonstrated that this mechanical processing does not affect spore germination rates (Claridge et al., 1992; Colgan and Claridge 2002). The slurry was filtered, centrifuged, and spore densities were estimated by using the previously described staining techniques. Spores that did not pass through the digestive tract (control) were handled with similar staining procedures to the spores in the feces.

In the case of the MTT staining technique, six tubes containing the following solution were prepared: a sample of 5 ml (feces dissolved), 1 ml of reference buffer, 1 ml of succinic acid and 1 ml of MTT. The spores were incubated for 1–2 hours at 37°C in water. To each sample tube we added one aliquot then placed it in a Neubauer chamber (Torres, 1992; Sot et al., 1996). Quantification of active spores was made by bright-field optical microscopy. For the hematoxylin staining technique (Santiago-Martínez and Estrada-Torres, 1999), the nuclei of cells become intensely stained because these contain nucleic acids that oxidize the hematoxylin to form a substance purple to dark purple called hematein (Montuenga et al., 2009). In the case of viable spores, nuclei are stained reddish or violet. Solutions of the feces were placed in a 1:3 ratio of glacial acetic acid/absolute alcohol remaining during three days, followed by centrifuging for five minutes at 2,500 rpm and removing the supernatant. Subsequently, we added 10 ml of 95% ethyl alcohol to the obtained solutions, stirred and centrifuged again, equating weights of all samples to 68.9 g. This process was repeated once more. The sample was then hydrolyzed with the addition of 3 ml of absolute alcohol and the following solution: 0.1 g AlNH4(SO4)2·12H2O, 0.1 g CrK(SO4)2·12H2O, 0.1 g iiodid acid, and 3 ml of concentrated hydrochloric acid. After mixing, the sample was left to stand for 12 minutes to allow complete hydrolysis and this was followed by centrifugation for 5 minutes at 2,500 rpm. The supernatant was removed and the spores were transferred to a fixative containing absolute ethanol, chloroform and glacial acetic acid (6:3:1 respectively), followed by centrifugation. Finally, the spores were transferred to the stain containing 1.0 g of hematoxylin in 50 ml of propionic acid to 45% and 0.25gr Fe (NH4)2(SO4).12H2O. To assess the number of viable spores, the samples were analyzed using the same protocol as with MTT staining (Torres, 1992).

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1.5 Statistical analysis

The response variable analyzed was the proportion of active and viable spores following mice gut passage, by specifying a generalized linear model (GLM) with family = binomial, using the computer program R (Development Core Team: www.r-project.org) (Crawley, 2007). Binomial errors are used when we know the number of successes as well as the numbers of failures; in this case, the number of active and no active spores; and the number of viable and non viable spores. Because the data were overdispersed we used a quasibinomial model to account for the overdispersion, using an F test instead of a chi-squared test to compare the complete and simplified model (Crawley, 2003, 2007).

The complete GLM included the fixed factors “consumer treatment” (i.e., consumed by Peromyscus alstoni or P. maniculatus, and controls), “fungal species” (i.e., Suillus and Laccaria) and the interaction of the two main effects. Simplified models were chosen using the backward elimination of non-significant factors (Crawley, 2007). The significance of a particular factor was based on the change in deviance between the full and simplified models. When a significant effect was detected overall, a posterior t-test was used to determine differences among either main factors or their interactions. A Bonferroni adjustment was done for adjusting alpha to multiple comparisons (n = 5 comparison, adjusted alpha = 0.01).

2 Results

2.1 Spore activity

Fungi offered to mice specimens were consumed entirely in all cases. The proportion of active spores was not significantly different from controls either after mouse-gut passage ($F_{2, 34} = 2.68, P = 0.09$) or between the two fungal species ($F_{1,32} = 3.03, P = 0.08$). However, the interaction was significant ($F_{2, 30} = 14.37, P < 0.001$) indicating higher spore activity for S. tomentosus after gut passage by both rodent species relative to the control ($t = 3.22, P = 0.007$, Fig. 1a). Irrespective of fungal species, no differences were found in spore activity between rodent species ($t = 0.85, P = 0.42$, Fig. 1a). This contrasted with findings for L. trichodermophora, where in the proportion of active spores of this fungal species was lower than the control treatment for both species of mice ($t = 5.53, P < 0.001$, Fig. 1a). No differences were found between rodent species for L. trichodermophora ($t = 0.47, P = 0.63$, Fig. 1a).

2.2 Spore viability

The proportion of viable spores was significantly different from controls for both mouse-gut passage treatment ($F_{2,34} = 32.02, P < 0.001$) and fungus species ($F_{1,32} = 18.52, P < 0.001$). Similarly, the interaction was significantly different ($F_{2,30} = 8.51, P < 0.001$). For both fungal species there was a decrease in the proportion of viable spores in comparison with the control ($t > 4.8, P < 0.001$) (Fig. 1b).

3 Discussion

We found that passage through the gut of either species of rodent had a significant effect on spore activity and viability for both fungal species. The proportion of active spores of L. trichodermophora in the feces of both species of rodents was less than that recorded for the control and the proportion of active spores of S. to-
mentosus in the feces of each species of rodent was higher than in the control. The viability of spores was lower for both fungi when consumed by either rodent relative to the controls.

Spores dispersed into the air by fungal sporocarps produce a scattered and diffuse spore rain over long distances, resulting in many spores arriving to sites lacking roots of suitable mycorrhizal host plants (Allen et al., 1992). Such obstacles to effective dispersal of a fungus species are compensated to some degree by vertebrate consumption of sporocarps which can result in very different dispersal patterns. For example, the establishment of ectomycorrhizal fungi in early successional habitats may take place through rodent dispersal to these sites, and without the rodents this would otherwise not occur. In addition, fecal pellets represent a concentrated ‘spore packet’ that is deposited on the soil surface at specific distances from the site where fungi are consumed (Johnson, 1996; Maser et al., 2008).

Post-digestion, the viability of hypogeous spores is known to remain consisten or even to increase (Kotter and Farentinos, 1984; Colgan and Claridge, 2002; Maser et al., 2008). As such, the spores these have been shown to successfully establish mycorrhizal interactions with seedlings (Kotter and Farentinos, 1984; Castellano and Trappe, 1985; Miller, 1985; Cork and Kenagy, 1989; Claridge et al., 1992, 1999; Colgan and Claridge, 2002; Ashkannejhad and Horton, 2005). Although epigeous sporocarps are usually a food source for obligate (e.g., rat-kangaroo and voles) and preferential mycophasists (e.g., squirrels) (Fogel and Trappe, 1978; Maser et al., 1978; North et al., 1997), as well as opportunistic species such as deer mice and chipmunks (Maser et al., 2008), studies about how fungal consumption by these animals affect spore activity and viability is still lacking.

Spores of epigeous fungi (L. trichodermophora and S. tomentosus) consumed by two species of rodents in Mexico showed intact nucleic and active mitochondria, which indicates a potential for germination after passage through their digestive tracts. Results obtained from the feeding trials showed that passage through the digestive tracts of both rodents resulted in a significant increase in the proportion of active spores of S. tomentosus (0.64 in P. alstoni and 0.73 in P. maniculatus) in relation to active spores quantified for the control treatment (0.40). Nonetheless, the opposite pattern was observed in terms of spore viability for this fungal species, as controls exhibited the highest average value (0.82). In spite of this latter finding, both deer mice and volcano mice showed a mean value of spore viability for S. tomentosus greater than 50% (0.60 to P. alstoni and 0.69 to P. maniculatus). On the other hand, after digestive tract passage, L. trichodermophora spores had lower values of active (0.37 in P. alstoni and 0.40 in P. maniculatus) and viable spores (0.26 in P. alstoni and 0.30 in P. maniculatus) than controls (0.82 active and 0.90 viable spores, respectively).

Although showing lower values compared with controls, the activity and viability of spores quantified for both fungal species showed a tendency towards higher values in P. maniculatus than in P. alstoni. This result is important if we consider the behavioral patterns of both species. For example, at La Malinche deer mice are found in pine-fir forest remnants where they inhabit subterranean burrow systems and fallen trees. Usually this species moves among forest patches, enhancing spore dispersal of mycorrhizal fungi to isolated trees (Durán, 2006). By contrast, although the volcano mice had lower values of activity and viability of spores than deer mice, this species usually inhabits pine forest areas and grasslands that have undergone regeneration after fires, thus highlighting the potential importance of this rodent species for spore dispersal to open areas and presumably favoring the establishment of tree and grass saplings (Carlos Lara, unpublished data. Laboratorio de Ecología de la Conducta, Centro Tlaxcala de Biología de la Conducta, Universidad Autónoma de Tlaxcala).

Findings from this study agree with previous findings, which have demonstrated that spores of some mycorrhizal fungi species remain viable after undergoing passage through the digestive tract of mycophasing mammals (Colgan and Claridge, 2002). Although our data showed that gut passage acts as a trigger for sporal activity, there are other studies which have showed a reduction in these parameters following gut passage (see review by Colgan and Claridge, 2002). We suggest that such contrasting results may be due to anatomical and microbiota differences in digestive systems among mice species and/or to variation in spore residence time in the guts. Further research is needed to address these conditions, as well as their significance under natural conditions.

Findings for spore activity and viability values after gut passage for both rodent species may be considered

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1 Miller SL, 1985. Rodent pellets as ectomycorrhizal inoculum for two Tuber spp. VI North American Conference on Mycorrhizae, Oregon.
small loads of spores that have may represent an effective mechanism for dispersing that the dispersal of spores by deer and volcano mice promote a differential effect of mycophagy, suggesting sporocarps with high numbers of spores. These features values are similar to those observed for controls of L. trichodermophora in this study.

The relative importance of spore dispersal via gut passage through rodents versus aerial dispersal from epigeous fruting bodies has not been quantified at La Malinche. However, preliminary observations suggest that rodent dispersal is common for these fungi, given high levels of rodent grazing at sites where these fungi are found, as well as the high diversity of rodent species involved (Durán, 2006). Recently, inoculation experiments with spores of both species of fungi showed that mycorrhization increases when spores are obtained from feces of both species of mice (Citlalli Castillo-Guevara, unpublished data, Laboratorio de Sistemática, Centro de Investigación en Ciencias Biológicas, Universidad Autónoma de Tlaxcala). L. trichodermophora and S. tomentosus species are quantitatively important taxa in many ectomycorrhizal communities (Gardes and Bruns, 1996; Taylor and Bruns, 1999), although both genera have contrasting life histories. At La Malinche, México, Laccaria genus produces small ephemeral sporocarps with few spores and Suillus genus produces large sporocarps with high numbers of spores. These features promote a differential effect of mycophagy, suggesting that the dispersal of spores by deer and volcano mice may represent an effective mechanism for dispersing small loads of spores that have L. trichodermophora, and/or promote that large quantities of S. tomentosus spores, mostly dispersed by wind, arrive at specific locations in the forest. Although the generality of these mechanisms remain to be tested using other epigeous fungal species and rodent species, they point to the need for understanding the biotic linkages driving fungal community structure. An important follow-up study would be to relate fungi use to availability (abundance to sporocarps) in the natural environment. This would lend support (or refute) the hypothesis that the mice are dispersing spores intentionally or passively through the forest.

Any factor that disrupts these food webs, such as the introduction of invasive pests (e.g., feral cats or dogs), and/or changing land use by transforming forest areas to cropland, could alter patterns of gene flow, fungal populations and community structure, which in turn could influence forest ecosystem function through altered mycorrhizal interactions. A greater understanding of the role of opportunistic mycophagous rodents in structuring populations and communities of mycorrhizal fungi will enhance our ability to manage and conserve the biodiversity and function of such communities.

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