Experimental infection of almond trees seedlings (*Terminalia catappa*) with an environmental isolate of *Cryptococcus neoformans* var. *gattii,* serotype C

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Summary

Recently, our laboratory reported the isolation of *Cryptococcus neoformans* var. *gattii,* serotype C for the first time from almond trees (*Terminalia catappa*) detritus. The aim of the present study was to establish the survival of *C. neoformans* in almond trees seedlings. Thirty seedlings were infected in the stems and samples were taken and processed at different times and by different techniques. No morphological alterations (macro or microscopic) were observed in the infected plants. However, *C. neoformans* was found to be viable for at least 100 days after infection. These data constitute our first approach towards the understanding of the yeast interactions with a host-plant.

Key words *Cryptococcus neoformans*, Ecology, Almond trees

Infección experimental de plántulas de almendro (*Terminalia catappa*) con un aislamiento ambiental de *Cryptococcus neoformans* var. *gattii,* serotipo C

Resumen

Recientemente, nuestro laboratorio informó por primera vez el aislamiento de *Cryptococcus neoformans* var. *gattii* serotipo C a partir de detritos de almendros (*Terminalia catappa*). El objetivo del presente estudio fue evaluar la supervivencia de *C. neoformans* en plántulas de almendros. Treinta plántulas fueron infectadas en el tallo; el material vegetal fue procesado con diferentes técnicas y en diferentes periodos postinfección. No se observó alteración macroscópica ni microscópica en las plántulas infectadas. Sin embargo, *C. neoformans* permaneció viable hasta 100 días después de la infección. Estos datos constituyen una primera aproximación para comprender la relación del hongo con una planta hospedera.

Palabras clave *Cryptococcus neoformans*, Ecología, Almendros

Two varieties and five serotypes are described for the pathogenic yeast *Cryptococcus neoformans*: *C. neoformans* var. *neoformans* (serotypes A, D and AD) and *C. neoformans* var. *gattii* (serotypes B and C). The two varieties show marked differences in their habitat, epidemiological risk factors and geographic distribution, as well as in their virulence [1].

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C. neoformans var. *neoformans* habitat has been closely related with soil enriched with bird excreta and sporadically, also with plant material [1,2]. In contrast, *C. neoformans* var. *gattii* (serotype B) habitat has been associated more regularly with plant material, especially with *Eucalyptus* spp. and other tropical trees [3-9]. On the other hand, *C. neoformans* var. *gattii,* serotype C has been exclusively recovered from almond trees (*Terminalia catappa*) detritus in Colombia [10]. This finding prompted us to study in more detail the association of the yeast with almond trees, and to evaluate the survival of the yeast in plant tissue. The corresponding preliminary data is presented in this report.

For this purpose, we infected 30 almond trees (*T. catappa*) seedlings, 45-50 cm high, with either 10^7 or 10^6 colony forming units (CFU) of *C. neoformans* var. *gattii,* serotype C (INS-755, recovered from an almond tree detritus), contained in 20 µl and distributed in two doses of 10 µl given in a 30 min period. Twenty seedlings were inoculated with one incision in the stem, 15 with 10^7 CFU and 5 with 10^6 (group 1). The remaining 10 with 10^6/incision in four incisions (group 2), all done with a surgical blade. Upon absorption of the inocula the inci-

sions were covered with liquid paraffin. All seedlings were maintained in the laboratory at a mean temperature of 19 °C for 100 days as the final observation period.

Different techniques for processing plant samples and attempting fungal visualization in the corresponding tissues were evaluated. In group 1 leaves, roots and also the seedling's stems were processed by maceration with mortar and pestle. Group 2 plants were processed according to the technique described for endophytic fungi, which consists in directly plating small stem pieces into appropriate culture media [11] which in our case was the *Guizotia abyssinica* agar. The soil was processed using standard techniques [10].

For microscopic visualization of the yeast, 3 histological stains were used: hematoxylin-eosin, Grocott and fuchsin fast green; fresh preparations with distilled water were also done from stem fragments collected at time of bark processing for the endophytic fungi technique.

In group 1 seedlings (inoculated with 10^7 CFU), the fungus was recovered from the leaves in two of the 11 plants studied up to 40 days post-inoculation. In the stems, this period was longer, with fungal recovery up to 60 days in three of the seven plants studied. In contrast, leaves from group 1 seedlings (inoculated with 10^6 CFU) gave rise to positive cultures in one out of five plants up to 40 days. As for stems only one of the five plants in this group allowed isolation of the fungus only at 40 days. Neither soils nor roots proved positive during the observation period.

In contrast, in group 2 seedlings processed as described for endophytic fungi, the yeast was recovered from the stems at 60 and 100 days, post inoculation, 1/1 and 9/9 respectively. The yeast was never recovered from leaves, roots or soil samples.

The Grocott stain proved to be the best means for fungal observation in plant tissues and the results that follows were obtained from the corresponding observations. A semi-quantification of the yeast in the tissue was done taking into account the number of positive slides and the location of the yeast either in the cortex or in the pith (Figure 1). From the seedlings in group 1, inoculated with 10^7 CFU, 52% (25/48) revealed the presence of the fungus. From those inoculated with 10^6 CFU, only 29% (8/28) were positive. With both inocula, the fungus was located preferentially in the bark.

In group 2, the fresh, distilled water preparations from stem fragments collected at time of processing by the endophytic fungi technique revealed that 59/64 (92%) fragments harbored the fungus even after 100 days post-inoculation (Figure 2).

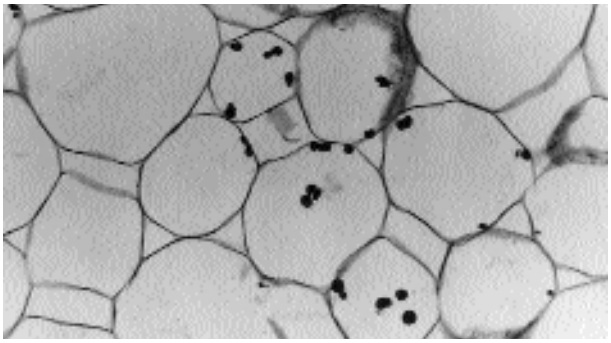

Figure 1. Section from stem inoculated with *Cryptococcus neoformans* var. *gattii* serotype C. Yeast cells are observed in the cortex, 60 days after infection. Grocott staining (100X).

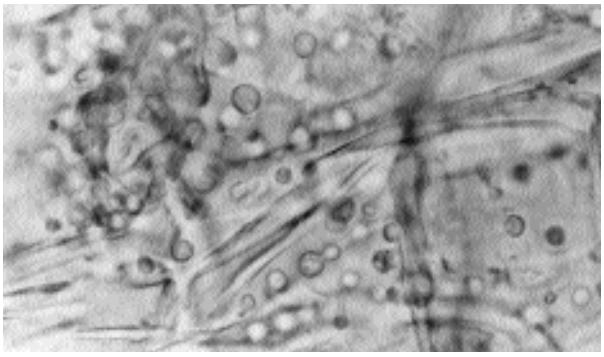

Figure 2. Fresh preparation with distilled water of an infected stem. Longitudinal section. Numerous yeast cells are observed (100X).

The latter result, this is, survival of *C. neoformans* var. *gattii* in the seedlings for such an extended period is of importance and could be interpreted as an adaptation of the fungus to the plant. However, it would be very important to determine if the survival time could be expanded to longer periods, in order to describe in detail the association established between the host and the fungus.

The best processing technique for recovering the fungus from plant material was the one described for endophytic fungi. Additionally, the fresh water preparation proved to be an adequate alternative for yeast visualization in plant tissue while the Grocott stain was be very useful in locating the yeast in tissues. An important observation of this preliminary work was the absence of macro or microscopic alteration in the host tissues infected with *C. neoformans*, raising the possibility of the plant being a transitory host to the fungus.

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