NOTE

A Technique for the Introduction of Fungi to Bark Beetle Mycangia[1]

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Bark beetles (Coleoptera: Scolytidae) and ambrosia beetles (Coleoptera: Platypodidae) are often closely associated with symbiotic or mutualistic fungi (Batra, 1966. Science 153: 193-195; Beaver, 1989. pp. 121-143, *In* N. Wilding, N. M. Collins, P. M. Hammond, J. F. Webber (eds.), Insect-Fungus Interactions. Academic Press, London). The associated fungi may be carried in specialized cuticular structures, termed mycangia, that can take a variety of forms (Francke-Grosmann, 1967. pp. 141-205. *In* S. M. Henry (ed.), Symbiosis Vol. 2., Academic Press, New York). Several species of *Dendroctonus* bark beetles possess thoracic (e.g., *D. brevicomis* Le Conte and *D. frontalis* Zimmermann) or maxillary (e.g., *D. ponderosae* Hopkins and *D. Jeffreyi* Hopkins) mycangia for carrying symbiotic fungi (Francke-Grosmann, 1967. pp. 141-205. *In* S. M. Henry (ed.), Symbiosis Vol. 2., Academic Press, New York; Whitney and Farris, 1970. Science 167: 54-55; Barras and Perry, 1971. Ann. Entomol. Soc. Amer. 64: 123-126; Happ et al. 1971. Tissue and Cell 3: 295-308). Usually only one or two specific fungi are carried in a mycangium to the exclusion of many other species present both on the exterior of the beetles and in the wood in which the beetles are found. The mycangia are often associated with glandular cells, and it has been suggested that secretions produced by these cells may be responsible for the selective culture and nutrition of fungi (Francke-Grosmann, 1967. pp. 141-205. *In* S. M. Henry (ed.), Symbiosis Vol. 2., Academic Press, New York; Happ et al., 1971. Tissue and Cell 3: 295-308; Barras and Perry, 1972. Z. Angew. Entomol. 71: 95-104; Whitney and Cobb, 1972. Can. J. Bot. 50: 1943-1945; Paine and Birch, 1983. Environ. Entomol. 12: 1384-1386). Mycangial fungi are acquired in the pupal chamber by newly-eclosed adult beetles prior to emergence from the host tree (Whitney, 1971, Can. Entomol. 103: 1495-1503; Paine and Birch, 1983. Environ. Entomol. 12: 1384-1386).

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There has been considerable interest in understanding the evolutionary and ecological relationships between beetles and associated fungi (Schowalter and Filip 1993. Beetle-Pathogen Interactions in Conifer Forests, Academic Press, London. 252 pp.). However, it has been difficult to separate the effects of mycangial and non-mycangial fungi on the fitness of the beetles. Prior techniques developed for producing fungus-free beetles had drawbacks since they either incorporated antibiotics into the diet (Marler and Barras, 1978. J. Georgia Ent. Soc. 13: 121-124) which can have adverse physiological effects on the beetles (Greenburg, 1970. Bull. Entomol. Soc. Am. 16: 31-36), or did not allow for a period of maturation feeding (Barras, 1973. Can. Ent. 105: 1295-1299). Most importantly, these techniques did not allow fungus-carrying beetles to be produced in the same manner as fungus-free beetles. Differences in the way beetles develop can seriously alter the outcome of experiments designed to test for effects of fungi on beetle fitness. The objective of our research was to develop a method to produce both fungus-free beetles and beetles associated with specific mycangial fungi without the use of antibiotics and which allowed maturation feeding. The technique was used successfully to examine the relationships between two sibling species of bark beetles, the mountain pine beetle, *D. ponderosae*, and the Jeffrey pine beetle, *D. jeffreyi,* and their closely-related mycangial fungi. Both sexes of these beetles possess mycangia located on the maxillary cardines (Whitney and Farris, 1970. Science 167: 55-55; TDP, pers. obs.).

Pseudo-pupal chambers (PPC) for the beetle pupae were made from uninfested bark of host pines. Trees with minimal phloem thickness of 3 mm were felled and cut into bolts for use within 4 to 5 weeks. The outer bark was shaved to an approximate thickness of 9 mm using a drawshave just prior to use in the experiments. The bark was then cut into squares (approximately 3 cm^2) using a bow saw to cut through the bark to the sapwood. Bark squares were removed from the bolt by lightly tapping a chisel under one edge of the bark. A 10-mm diam cork borer was used to cut a hole into the phloem in the center of each square but which did not extend completely through the bark, thus creating a "pseudo-pupal chamber." The squares were autoclaved for 45 min to sterilize them. The squares were then placed in tightly sealed plastic bags and frozen until needed. Just prior to use, the squares were wrapped in foil in batches of 10 and autoclaved a second time to assure they were sterile.

Water ager (2%) was prepared, poured into Petri dishes, and allowed to stand 7 to 14 days until no condensation was apparent in the plates. Using sterile techinque to avoid contamination of plates with airborne fungi, a square of sterilized bark was placed into each plate with the PPC opening upward. A tight fit between the top surface of the square and the Petri dish lid prevented adult beetles from leaving the PPC prematurely after eclosion. Surface-sterilized pupae (Barras, 1972. J. Econ. Entomol. 65: 1504) were placed individually into PPCs to produce fungus-free adult beetles. If beetles were to acquire a fungal associate, the desired fungus was inoculated by placing a small plug of media containing an active culture into the PPC 1 wk prior to the introduction of the pupae. Thus, the fungi grew sufficiently to produce fruiting structures in the PPC by the time the beetle had eclosed to the adult stage.

After a pupa had been placed in the PPC, the dish was closed and sealed using a strip of Parafilm™. A slit extending around approximately 1/3 of the circumference of the dish was cut in the Parafilm™ seal under the lip of the dish cover. This allowed for gas exchange and prevented condensation within the dish. Dishes were checked daily for excess condensation and contamination. If a plate developed excess condensation, the Parafilm™ was removed for a few days and then replaced.

When the beetles eclose, they are tan in color (callow), soft-bodied, and not yet sexually mature. Callow adults were left in the PPC for about 2 wks to allow the exoskelton to darken and harden, to allow maturation feeding on the phloem and fungi of the square, and to allow acquisition of the fungi in the mycangium. Callow adults sometime exited the square prematurely and became stuck on their backs on the agar in the bottom of the plate. When this happened, the plate was opened and the beetle placed back into the PPC using sterile forceps.

This technique successfully produced fungus-free beetles of both *D. ponderosae* and *D. jeffreyi*. Adults of each species were produced that carried their specific mycangial fungi or that of the sibling species. An attempt to introduce a *Penicillium* sp. (not normally carried in the mycangium) to mycangia of ten *D. jeffreyi* was unsuccessful. The surface of the beetles was contaminated with the *Penicillium* sp., but the mycangia remained free of fungi, suggesting that the mycangia, to some degree, are selective.

The mortality rate of pupae that had been surface sterilized and introduced to PPCs was, on average, less than 25%. Some mortality (< 10%) also occurred during eclosion to the adult stage. After ecolsion, mortality was low (< 2%). Dissections of mycangia of beetles developing with fungi (n = 50) found 100% acquired the fungi in the mycangia.

This technique can produce adults for use in experiments designed to test for effects of mycangial and other non-specific subcortical fungi on beetle fitness and to determine the specificity of mycangial associations. These investigations will increase the understanding of bark beetle/fungus associations, in particular, and symbiotic associations, in general, and to test hypotheses that were not previously technically possible.