



Armillaria collection methods: tips and tricks

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Introduction

This report is aimed toward improving general awareness of various types of *Armillaria* samples and recommended survey practices to obtain collections for isolation/culture, DNA-based identification, and/or future studies. High-quality samples yield a higher success rate for DNA-based identification and allow isolation in the laboratory to obtain pure, living *Armillaria* cultures that can be archived for long-term storage for use in future research projects. In root disease surveys, samples of pathogenic *Armillaria* are frequently obtained from trees showing symptoms of disease, such as yellowing needles (Fig. 1A), thinning crown, basal resinosis (Fig. 1B), upturned root balls/wads (Fig. 1C), or recently “red dead” needles (Fig. 1D). However, above-ground symptoms likely only represent the “tip of the iceberg” as it relates to *Armillaria* in both a pathogenic and non-pathogenic state. In most cases, *Armillaria* can cause reduced growth and root disease in trees that show no obvious above-ground symptoms (Cruickshank 2011). Alternatively, *Armillaria* exhibiting little, or no signs of pathogenicity is also of interest for surveys and research studies. **Armillaria is most often present as rhizomorphs on trees that appear to be perfectly healthy.** For example, rhizomorph-producing *A. altimontana* that exhibited little/no sign of pathogenicity has been associated with improved health of western white pine (*Pinus monticola*) in northern Idaho, USA (Warwell 2019). In contrast, studies and empirical observation suggest that various disturbances, such as stress from other pathogens, insects, fire, soil compaction, tree thinning/harvesting, fertilization, extreme weather, climate change, etc., may “trigger” *Armillaria* root disease (McDonald 1987a, Woods 1994, Lundquist 2000, Kim et al. 2010, Glaeser and Smith 2013, Kubiak et al. 2017). This interaction can be evident with insect attack, where stress due to insect damage may induce pathogenicity from pre-existing *Armillaria* rhizomorphs on the roots. A reciprocal relationship is also common in which bark beetles may be more attracted to stressed trees predisposed by *Armillaria* root disease (Tkacz and Schmitz 1986, Lalande et al. 2020, Sierota and Gradzki 2020). Studies in northern Idaho, USA found that 92-96% of trees attacked by bark beetles also had root disease (Hertert et al. 1975, Kulhavy et al. 1984).

A general *Armillaria* survey method is to make collections from trees within random, 0.04-hectare plots (a circular plot with a ca. 11.3-m or 37-ft radius) (McDonald et al. 1987b, 2011). Typically, three trees of different size classes for each species are surveyed/sampled for all *Armillaria* collection types (rhizomorph, mycelial fan, colonized wood, fruiting body), with rhizomorphs being the most frequent collection type. Samples from different shrub species are also surveyed/sampled. With this sampling strategy, habitat type and plant association information are also recorded to determine relationships with *Armillaria* species. This random-plot survey strategy is time consuming and may require a crew of three to five people to sufficiently survey three to four plots a day. Recently, survey studies have shifted towards using bioclimatic modeling of *Armillaria* species distribution using algorithms, such as MaxEnt (Klopfenstein et al. 2009, Kim et al. 2021, 2023). For these bioclimatic modeling

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studies, single-point collections can be used, which require fewer people and less time to collect sufficient data. An important consideration for bioclimatic modeling studies is to include diverse samples of each *Armillaria* species across its potential geographic range, which represent variable climates. A major advantage of using MaxEnt over other algorithms is that it excels at using presence-only data, because it is impractical and cost prohibitive to confirm absence data for *Armillaria*.



Fig. 1. 1A: Douglas-fir (*Pseudotsuga menziesii*) with yellowing needles adjacent to healthy, green-needed trees.

1B: Tree shown in 1A displaying signs of basal resinosis. 1C: Root ball/wad of a “red dead” bigleaf maple (*Acer macrophyllum*). 1D: Forest with high percentage of “red dead” conifers. Photo credits 1A, 1B: J.W. Hanna, Chicago Peak, Rogue River – Siskiyou National Forest (NF), California, USA; 1C: J.W. Hanna, Ventana Wilderness, Los Padres NF, California, USA; 1D: J.W. Hanna, The Whaleback, Klamath NF, California, USA

Safety

When collecting *Armillaria* samples, safety must be considered at all times. Approach hazard trees or any tree that may be compromised with root disease with caution and when in doubt error on the side of caution. Only collect samples if it is safe to do so and consider skipping the collection or finding an alternative collection site if necessary. See Fig. 2A, 2B, 2C, and 2D for additional safety tips to consider when working in the field.



Fig. 2. 2A: Poison oak rash and leaflets (inset) 2B: Black bear. 2C: Northern Pacific rattlesnake. 2D: Weathered road signs. Photo credits: 2A: J.W. Hanna, Clarkston, Washington, USA 2A (inset): David Cappaert, Bugwood.org 2B: J.W. Hanna, Latour State Forest, California, USA 2C: J.W. Hanna, Barlow Flat, Ventana Wilderness, Los Padres National Forest (NF), California, USA; 2D: J.W. Hanna, Rouge River – Siskiyou NF, California, USA

Sample labeling

In general, sample labeling of *Armillaria* should reflect collector name, site name, sample number, and letter for collection type: R=rhizomorph, B=basidiocarp, F=mycelial fan, and W=wood including zone lines or decay. For example, a collection from Sykes Campground may be labeled Sykes Camp 1R for a rhizomorph and Sykes Camp 1F for a mycelial fan from the same tree. A rhizomorph sample from a second tree or shrub from this general area would be labeled Sykes Camp 2R. Other information that should be collected include date of collection, GPS coordinates (preferably in decimal degrees); host species and status (e.g., live and healthy, red dead, old snag, etc.), and photos; AK = thought to be *Armillaria* killed, DBH, other notes, such as yellowing needles/leaves, thinning crown, resinosis, etc.; and any special notes about the site in general.



Fig. 3. 3A: *Armillaria solidipes* basidiocarps on an alder (*Alnus* sp.) snag. 3B: *A. sinapina* basidiocarps. 3C: *A. gallica* basidiocarps. Photo credits 3A: J.W. Hanna, La Poel, Washington, USA; 3B: J.W. Hanna, Birch Bay, Washington, USA; 3C: J.W. Hanna, Heaps Peak, San Bernadino National Forest, California, USA

Basidiocarps

Armillaria basidiocarps (aka “honey mushrooms”) are the collection type most frequently encountered and recognized by mycologists and naturalists (amateur and professional). Keys are available to assist identification of *Armillaria* species based on basidiocarp morphology (most notably Volk, https://botit.botany.wisc.edu/toms_fungi/armkey.html). However, morphological keys typically do not include species, such as *A. altimontana*, which can be difficult to distinguish from species with similar morphology (Brazee et al. 2012). Furthermore, morphological keys may not account for intraspecies variation that occurs across North America. For example, despite the fact that basidiocarps sometimes appear to contrast greatly (Fig. 3A and 3B), experienced mycologists were unable to find consistent morphological differences among basidiocarps of *A. solidipes* and *A. sinapina* in western North America (McKenny et al. 1987). DNA sequencing of the translation elongation factor-1 alpha (*tef1*) gene is currently the easiest and most reliable way to identify all ten *Armillaria* species and one *Desarmillaria* species that are currently recognized in North America (Ross-Davis et al. 2012, Elías-Román, et al. 2018, Antonín et al. 2021). Fresh basidiocarps, preferably small caps that are collected just before the basidiocarp gills start to show (Fig. 3C), are ideal for isolation, culturing, and DNA-based identification techniques. Alternatively, basidiocarps can be gently dried in a dehydrator, but these samples only have potential for DNA- and/or morphology-based identification because they cannot be cultured and/or stored as living archives. Generally, basidiocarps are most abundant in North America during the fall/winter months from October to February with a later occurrence in southern and/or lower elevation areas.



Fig. 4. 4A) *Armillaria* rhizomorph size compared with knife for reference at various magnifications. Note: missing parts of the outer rhizomorph sheath reveal white mycelial cords on the inside. 4B) Example of roots (top) vs rhizomorphs (bottom). 4C) Rhizomorphs extending above the soil line. 4D) Rhizomorphs revealed under bark of dead big leaf maple (*Acer macrophyllum*). 4E) The rhizomorph stretch test. 4F) Rhizomorph collection tools. 4G) J.W. Hanna savoring the “mushroomy” scent of a freshly dug “rhizo”. Photo credits 4A, 4B: J.W. Hanna, Brushy Creek, Rouge River – Siskiyou National Forest (NF), California, USA; 4C, 4E: J.W. Hanna, Ventana Wilderness, Los Padres NF, California, USA; 4D: J.W. Hanna, The Whaleback, Klamath NF, California, USA 4G: P.I. Bennett, Yuki Wilderness borderlands, Mendocino NF, California, USA 4F: J.W. Hanna

Rhizomorphs

Armillaria rhizomorphs are fungal structures with the appearance of roots to the untrained eye (Fig. 4A, 4B, 4C, 4D, 4E, and 4G). *Armillaria* rhizomorphs are frequently present across many forest types in association with diverse woody hosts, whether or not they are exhibiting symptoms of *Armillaria* root disease. After practice digging for and collecting *Armillaria* rhizomorphs, surveyors will likely find that *Armillaria* is extremely prolific across many forest habitats. For scientific study, it is important to obtain fresh, viable rhizomorphs. Often, above-ground rhizomorphs are dried, dead, and/or contaminated with other fungi, which prevents or impedes the success of isolation and culturing (Fig. 4C and 4D). Fresh living rhizomorphs can be collected by careful root excavation. It is recommended that at least two main roots be carefully excavated using the grubbing end of a Pulaski (Fig. 4F) to a depth of up to 0.5 m (1.6 ft) and distance of 0.6 m (2 ft) from the basal bole; however, rhizomorphs are frequently found at deeper depths and sometimes on roots >2 m (several ft) from the host's bole. While excavating the roots, it is very important that extra care is taken to not disturb the upper (2-3 cm or 0.8-1.2 in) soil/litter layer surrounding the root. Excavation of this upper layer is best performed carefully by hand and/or with a knife, stick, rock, or similar digging tool. As the remaining soil is swept away (a kitchen vegetable brush may be useful), visual inspections are made for the root-like structures and determinations are made to identify these structures as roots or rhizomorphs (Fig. 4B). Below is a practical guide with some tips and tricks to train your eyes and other senses for distinguishing rhizomorphs from roots.

Rhizomorph field-identification guidance:

- 1) The exterior of mature rhizomorphs is usually jet black in color, but younger rhizomorphs can be shades of red/brown/tan.
- 2) The interior of a fresh rhizomorph is almost always white, but sometimes the interior can be stained with shades of tan/brown/purple/red.
- 3) The bleach test: place suspected rhizomorphs in a ~20% bleach solution and set aside for a couple minutes. After the bleach treatment, the exterior of fresh rhizomorphs will remain jet black with white mycelium through the center. If rhizomorphs are left in the bleach solution for too long, their outer black sheath will eventually disintegrate leaving only the white mycelium. After bleach treatment, roots become brownish, reddish, or bleached-out tan in color with no bright-white interior. Rhizomorphs that have undergone the bleach test in the field are typically not saved for culturing, but the bleach test is useful for training in rhizomorph identification (Fig. 4B). Note: Use safety precautions when using the bleach solution, which is also damaging to clothing.
- 4) The pull test (Fig. 4E): compared to roots, rhizomorphs will have a bit more stretch/elasticity and the outer sheath will start to crack to reveal the lighter colored mycelia that will continue to stretch until breakage. For this stretch test, start with a gentle pull and slowly increase the tension.
- 5) The end test: The ends of the outer covering of both rhizomorphs and roots can be gently pulled off. The mycelial tuft at the broken end of a rhizomorph will bend very easily, while broken roots with their outer covering removed will typically remain firm when touched.
- 6) The light test: Blow off the soil as well and possible from suspected rhizomorph or rinse it with water. Hold the sample in direct sunlight close enough to your eyes that allows focus on the fine detail of the black outer sheath. While slowly rotating the sample between your thumb and index finger, a rhizomorph will “glisten” from many reflective surfaces in prismatic colors as it is rotated, somewhat similar to a kaleidoscope effect (A.L. Smith, personal observation). Geral McDonald described this phenomenon as “many little diamonds shining in the light.” This effect can also be observed through a 10x hand lens under bright sunlight.
- 7) The smell test (Fig. 4G): For some, the smell test is perhaps the easiest test. Rhizomorphs have a distinct “mushroomy” aroma with some having a very strong scent. Investigations to understand species-specific scents and the associated variation are ongoing, but some *Armillaria* species have been distinguished using an electric nose (Baietto et al. 2010). It would be interesting to determine if dogs or pigs could be trained to hunt *Armillaria* and possibly even distinguish species.

Some evidence suggests that *Armillaria* species can be identified by rhizomorph size and branching patterns, but this method has not been investigated among all known *Armillaria* species within their associated geographic ranges (Morrison 2004).



Fig. 5. 5A, 5B: *Armillaria solidipes* mycelial fan and zone lines on fir (*Abies* sp.). 5C: *A. altimontana* zone lines on aspen (*Populus* sp.). Photos 5A, 5B: J.W. Hanna, Abajo Mountains, Manti-La Sal National Forest (NF), Utah, USA; 5C: J.W. Hanna, Jarbidge Wilderness, Humboldt NF, Nevada, USA.

Mycelial fans and zone lines

Mycelial fans and colonized wood with zone lines are two other collection types that can be used to isolate and establish pure *Armillaria* cultures in the laboratory (Fig. 5A, 5B, and 5C). Mycelial fans from trees that still show signs of life are a definitive sign of pathogenicity. Other signs of pathogenicity in relation to mycelial fans include a host response from the tree, such as resin-soaked mycelial fans or defensive growth reactions (Kim et al. 2023). *Armillaria* zone lines are areas of melanin synthesis and accumulation in fungal hyphae that are apparently formed when *Armillaria* competes with other decay fungi, different *Armillaria* species, or individuals of the same species (Morris et al. 2021). Compared to other collection types, success rates are lower for obtaining pure *Armillaria* cultures from colonized wood.

Sample storage and transport

Collected rhizomorph samples are typically placed into 15-ml screw-top tubes, but samples can also be placed in zip-lock bags. It is best to keep samples cool and dry during transport and shipment. Typically, ice packs in coolers are used for transport and shipping, while using extra barriers, such as zip-lock bags or paper bags inside an outer zip-lock or trash bag, to keep samples (e.g., basidiocarps, mycelial fans, and colonized wood samples with zone lines) separated. Elevated bottoms made to fit inside the coolers can help keep samples suspended above melted ice during collection trips. Fresh basidiocarps can be wrapped in several layers of paper towel, then placed in a small box or paper bag within a zip-lock bag. An exception to shipping samples overnight with ice packs is when basidiocarps are over-mature or could not be shipped in a timely manner. For this exception, the basidiocarp sample can be dehydrated before shipment. Dehydrated samples are often sufficient for use in DNA-based identification and are sometimes useful for morphological studies and/or species descriptions.

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