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A Pure Culture Technique for Studying the Infection Phenomenon of Oak Roots By Armillaria mellea

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A Pure Culture Technique for Studying the Infection Phenomenon of Oak Roots By *Armillaria mellea*[1]

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Abstract. Oak seedlings were grown in a pure culture of *A. mellea* in large test tubes. A layer of white quartz sand, in which an acorn was planted, covered a PDA medium containing rhizomorphs of the fungus. After four months growth, roots from the oak seedlings were extracted from the agar, washed, fixed in FAA, sectioned, stained, and examined microscopically. Abnormal areas of the tap roots showed epidermis demarcated by a black layer of suberized cells. One section showed this suberized layer broken by penetration of a foreign object. The cortex around this penetration point was disrupted and stained a dark brown color. The affected area was demarcated from healthy cells by a layer of cork, three cells thick. This technique will enable the development of more critical studies on this infection phenomenon.

One of the basic difficulties involved in tree root disease investigations is the lack of proper techniques to study the exact nature and degree of parasitism exerted by root pathogens. The shoestring root rot fungus, *Armillaria mellea* (Fr.) Quel., has been studied extensively since Hartig (1894) pronounced it a primary parasite on conifers and *Prunus* spp., and only a wound parasite on inactive tissue of oak.

Most workers agree that *A. mellea* can be an active parasite on conifers (Day, 1927*b*; Long, 1914; Woeste, 1956). The nature of parasitism on broad-leaved trees, especially *Quercus* spp., is undetermined. From evidence observed in the field, Long (1914) thought that the fungus could act as an active parasite on *Quercus* spp. Others have indicated, though, that this fungus is a secondary parasite. It infects oak only when the tree is weakened by a primary causal agent, whether it be a fungus, insect, or environmental factor (Day, 1927*a*, 1929; Robinson, 1927).

Hartig (1873) observed that infection on healthy plants occurred

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from nearby infected plants. Infection on tea plants in Nyasaland was recorded to be from contact with infected roots (Leach, 1939). Observations made in a black currant field in England also showed that infection started from diseased root contact (Marsh, 1952). Three avenues of infection on apple and prune trees have been observed (Zeller, 1926). These were at wounds on roots; at the point of contact with infected roots, where tissue was acted upon by a toxic substance excreted by the diseased root; and at the point of emergence of lateral roots. In conifers, rhizomorphs penetrated through an undamaged root (Hartig 1894).

Anatomical investigations on infected roots of coniferous trees indicated that infection can occur by direct penetration (Day, 1927*a*). The establishment of the infection, however, depended on a susceptibility factor, because changes in the host tissue suggested resistance. Similar studies carried out on spruce (Woeste, 1956) also showed that direct penetration of a rhizomorph occurred by mechanical and toxic action of the rhizomorph. Success of infection was determined by a third factor, probably resistance.

Artificial inoculation trials, using fungus cultures on agar and bran placed in soil near citrus roots, failed to cause infection. Infected wood buried in the soil did cause infection (Bliss, 1941). The potency of the inoculum and the temperature of the soil were suggested as the two factors responsible for the establishment of infection (Bliss, 1941, 1946). Investigations on effect of inoculum size and distance from inoculum indicated that the degree of infection by *A. mellea* decreased with decrease in inoculum size and increase in inoculum distance (Garret, 1956).

Artificial inoculations tests on *Pseudotsuga menziesii* (Mirb.) Franco growing in a pure culture of *A. mellea* on sand failed to show infection (Raynor, 1930). However, the presence of cankers on roots and stem was taken as evidence of a fungal attack being resisted. Insertion of myelclial cultures of this fungus in cuts made at the root-collar of two-year old *Quercus robur* L. seedlings, or placing infected wood near the cut, failed to produce infection (Reitsma, 1932). Thomas (1934) succeeded in obtaining infection on some woody and herbaceous plants, by burying wood inoculum segments in the soil near the roots of seedlings. From subsequent anatomical investigations he concluded that a rhizomorph can penetrate directly through the cork, as a single unit, by mechanical force. In the penetration of the subsequent layer, a dissolving power of the rhizomorph was evident. This was attributed to a toxic substance produced in its advance by the rhizomorph.

A. mellea is frequently found on oak stumps and roots, but whether this fungus is capable of direct penetration of healthy, living oak

roots and of establishing a parasitic relationship is not fully understood. An attempt was made to develop pure culture techniques to study this phenomenon further.

Materials and Methods

An isolate of *A. mellea* was obtained from a strand of rhizomorpha subterranea growing upon a living lateral root of a suppressed northern red oak (*Quercus rubra L.*) at Pilot Knob State Park, Forest City, Iowa. Stock cultures were maintained on PDA medium at 23° C.

Acorns were collected from northern red oak (*Q. rubra*) and northern pin oak (*Q. ellipsoidalis* E. J. Hill) during the summer of 1958. Preliminary germination trials showed that seemingly mature acorns collected directly from the tree (before shed to the ground), with the exocarp removed, germinated within a week.

Large pyrex glass test tubes, 40 cm. x 5 cm., were used for growing the oak seedlings in the pure culture of *A. mellea.* Each tube was filled to about half with a two percent PDA medium. Cotton was used to stopper the tubes. After autoclaving and setting, each tube was inoculated with an agar disc carrying an unbranched fragment of an *A. mellea* rhizomorph. At the end of a seven-day incubation period at 23° C, the top surface of the medium was layered with an inch of sterilized, white quartz sand. Upon this sand layer was placed an acorn with its exocarp removed and surface sterilized with a five percent Clorox^{R} solution for three minutes. The sand in the tube was moistened to saturation with sterilized water from a 10 ml. pipette. The lower half of the tube was wrapped with aluminum foil. The tubes were placed upright in cans and incubated in growth chambers adjusted to an alternating temperature of 20° C-30° C (Varghese and McNabb, 1959) and a relative humidity of 90 percent. During the first two weeks, the tubes were taken from the illuminated chambers every three days and three ml of sterilized water added to the sand. After this period, the sand was moistened with sterilized water only once a week. Macroscopic observations on growth of seedlings and rhizomorphs in the tubes were made during these periods (Figure 1).

After four months, the tubes were removed and placed in a steam oven. When the medium had liquified, the tubes were filled with warm water at 50° C. The complete contents of the tubes were emptied into a large dish of warm water. The sand and medium adhering to the roots were washed off. Macroscopic examination of the roots and stems of the seedlings were made before they were placed in FAA solution for subsequent anatomical investigations.

After the tissues were allowed to soak for 24 hours in FAA, cross sections of root material were made for microscopic examination. The sections were stained with either safranin and picro analine blue (Cartwright, 1929) or cotton blue in Lactophenol and were mounted in glycerine for microscopic study.

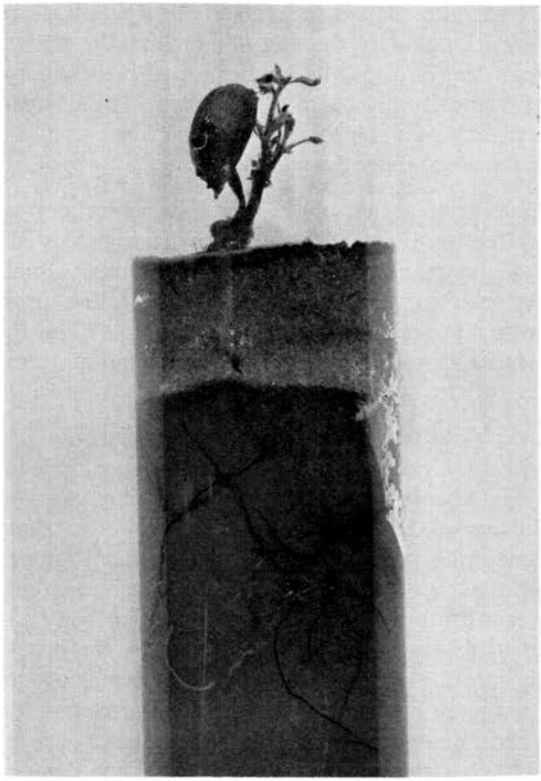

Figure 1. Oak seedling growing on a pure culture of *Armillaria mellea* (Courtesy of L. Facto).

Results

The rhizomorph fragment of the inoculum disc began producing branches within seven days after inoculation. By the end of four months rhizomorph branches commonly grew downward four inches in the medium and in a few cases extended five and six inches. The growth of the rhizomorphs was not vertically straight, but branched and twirled in all directions (Figure 1). However, no rhizomorph branches developed in the sand.

Approximately the upper two-thirds of the rhizomorphs had the dark brown color typical of the rhizomorpha subterranea. The grow-

ing regions were white, the browning being progressive from the growing trip towards the base.

The acorns planted on the sand layer began germination on the fifth day. Three seedlings died during the progress of the experiment. Those remaining produced roots, stem, and leaves. The leaves did not grow to full size; they grew to about one inch long, then began dying. From the buds at the axils of the dying leaf, a new shoot with leaves developed. By the end of four months the taproot had developed to a length of 3.0 to 3.5 inches, with the diameter at the root-collar varying from 0.20 to 0.35 inches. The growth in height of the stem varied from two to three inches. The cotyldons remained attached and were apparently undepleted.

Macroscopic examination of the roots showed abnormal taproots on three of the seedlings. The abnormalities consisted of warty growths, small lumps of tissue attached to the roots which probably were severed rhizomorph connections, and prolific production of lateral branches in some regions of the taproot. The roots of the other seedlings did not show any apparent abnormalities; the taproots had a straight tapering form.

Cross sections of the taproot made through the region of abnormality showed healthy xylem tissue in the center surrounded by phloem tissue and a large cortex area. However, in some cases the contour of the periphery of the epidermis was irregular. In these irregular areas the epidermis was well demarcated by developmnt of an abnormal black layer of suberized cells. It was apparent that this layer was formed around some intruding foreign body. In one section this suberized layer was broken through by the penetration of a foreign body into the cortex. The cells of the cortex around the area of penetration were apparently abnormal, disarranged, the walls of some cells disrupted; and the whole affected area was dark brown in color. However, this area was found to be well demarcated from the rest of the healthy cortex by a layer of cork, three cells thick, which formed a wall completely surrounding the outer periphery of the affected area. Cross sections of the normal regions of the root did not show any abnormal suberized cells near the epidermis, any discoloration in the cortex, or any secondary cork cell formation in the cortex.

Discussion

This study has developed a technique of growing oak seedlings on a pure culture of *A. mellea*. This, in addition to ensuring the specificity of the invading organism, also reduced the possibility of wounds on roots caused by mechanisms other than the organism under study.

The results of this study have indicated direct penetration by rhizomorphs of *A. mellea* into living oak roots. This does not agree with earlier field observations (Hartig, 1894) that infection of *A. mellea* on oak was only through wounds. Artificial inoculation studies on other woody plants have indicated direct penetration of living roots (Thomas, 1934).

The section which indicated good penetration into the cortex showed cells with brownish discoloration, as if a toxic substance had spread from the point of penetration. Other studies have indicated rhizomorph penetration of the cortex by mechanical means and a discoloration of cortical cells by toxic secretions of the fungus (Day, 1927*a*; Thomas, 1934).

The discolored area was isolated from the rest of the cortex by a layer of secondary cork, three cells thick. Such secondary cork cell formation has been reported in conifers (Day, 1927*a*; Rayner, 1930), and on other woody plants (Thomas, 1934). The cork formation observed in this study could have prevented establishment of infection. Cork formation, as evidence of infection being restricted, has been reported previously (Rayner, 1930). Whether such isolated penetration points can later establish infection by penetrating through the cork barrier when vigor (and therefore, resistance) of the tree is lowered is a matter that should be investigated further.

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