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# Mycelial lipids as an aid in identifying rust fungi in culture—*Cronartium fusiforme*

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The sterol and fatty acid content of mycelium from germinating basidiospores of *Cronartium fusiforme* was determined. The mycelium contained stigmast-7-enol, fungisterol, and possibly stigmasta-5,7-dienol. No ergosterol was detected. The mycelium contained the expected fatty acids and low relative proportions of 9,10-epoxyoctadecanoic acid. The absence of ergosterol, and presence of the epoxy $C_{18}$ acid and sterols typical of certain rust spores may be used for a relatively rapid confirmation of rust fungi in culture. Based on these chemical criteria, yeast-like cells isolated from the cultures of germinating basidiospores appear not to be *C. fusiforme*.

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On a déterminé la teneur en stérols et en acides gras des mycéliums de basidiospores en germination de *Cronartium fusiforme*. Le mycélium contient du stigmast-7-énol, fungistérol et probablement du stigmasta-5,7-diénol. On n'a pas décelé d'ergostérol. Le mycélium contient les acides gras usuels et de faibles quantités d'acide 9,10-époxyoctadécanoïque. L'absence d'ergostérol et la présence de l'acide époxy $C_{18}$ et de stérols typiques de certaines spores de la rouille peuvent être utilisées pour confirmer rapidement la présence de rouille en cultures. Selon ces critères chimiques, les cellules levuriformes isolées dans des cultures de basidiospores en germination ne semblent pas être *C. fusiforme*.

[Traduit par le journal]

Once considered obligate parasites, several species of rust fungi are now grown routinely in laboratory culture. Mycelia of these fungi are initially white but may develop the light orange pigmentation typical of rust fungi. Cultures of rust fungi exhibit relatively slow growth and in some cases produce spores or other reproductive structures characteristic of certain stages in the rust life cycle (Bushnell 1968; Hollis *et al.* 1972). The axenic culture of these fungi has been reviewed by Scott and McLean (1969). This study was conducted to determine whether mycelia and yeast-like cultures suspected of being *Cronartium fusiforme* have certain chemical characteristics that may serve to identify them as fust fungi.

Mycelial and yeast-like colonies suspected of being *C. fusiforme* Hedge & Hunt ex Cumm. were obtained in the following manner: young leaves of potted water oak seedlings (*Quercus nigra* L.) were misted with deionized water, dusted lightly with *C. fusiforme* aeciospores, and incubated in a growth chamber for 5 to 8 days at 20°C, and 100% relative humidity, with a 14-h photoperiod. Leaves were excised when uredial or telial pustules appeared and prior to epidermal rupture. Only hardened leaves showing abundant infections were selected. Ten to 20 leaves were cleansed gently with a mild detergent and rinsed thoroughly with deionized water. The leaves were then surface-sterilized by soaking for 5 min with constant agitation in 500 mL of a freshly prepared, filtered, saturated solution of calcium hypochlorite [$Ca(OCl)_2$] containing 0.5% Tween 20 and 1 mL of 1 *N* HCl. The leaves were aseptically removed from the $Ca(OCl)_2$ solution and 1 cm of the petiole end of each leaf was cut transversely. The leaves were then rinsed three times in 500 mL of sterile deionized water for 5 min with stirring.

The leaves were tested for contamination by placing them in deep petri dishes containing 100 mL of potato dextrose agar (PDA) (Difco) medium. The cut petiole end of the leaf was embedded in the medium and the leaf inclined at a 45° angle with the lower surface of the leaf facing the medium surface. These leaves were incubated for 72 h in a growth chamber under the conditions described above.

Uncontaminated leaves were transferred to deep petri dishes containing 50 mL of a medium

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containing (g/L): $KH_2PO_4$, 0.5; $K_2HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.014; $Na_2EDTA$, 0.019; sucrose, 30.0; yeast extract (Difco), 2.0; low salt acidicase (BBL), 4.0; and agar, 8.0. Oleic acid (10 mg/L) and sitosterol (1 mg/L) in 10 mL of ethanol were added to the medium while stirring vigorously. The medium was adjusted to pH 6.0 with 1 *N* KOH prior to autoclaving. The leaves were arranged in each petri dish as described above. An additional 5 mL of the medium without agar (broth) was added to each culture to provide a liquid surface layer. The leaves were incubated under a 14-h photoperiod at 20°C and a 10-h-dark period at 15°C. Small white mycelial colonies interspersed with infrequent small, yellowish, yeast-like colonies appeared on the medium surfaces after 7 to 12 days.

It was suspected that these colonies developed from germinating basidiospores expelled from telia on the lower surface of the oak leaves. Isolates were obtained from these colonies. Once established in culture, inocula for the mycelial cultures were prepared by grinding the mycelium in 10 mL of the broth. Each 250-mL Erlenmeyer flask containing 75 mL of the solid medium was inoculated with 1 mL of the inoculum. Yeast-like cells were suspended in the broth and 1 mL placed in each 250-mL flask containing solid medium as before.

After 5 days of growth, the mycelium was removed from the growth medium surface with a spatula. Yeast-like cells on the medium surface were suspended in water and collected by centrifugation. The fungal materials were washed, dried by lyophilization, weighed, ground in a mortar with pestle, and the lipids extracted for 1 h with chloroform: methanol (2:1 v/v). Cell debris was removed from the extract by filtration and the solvent was evaporated under nitrogen. Fatty acid methyl esters (FAME) were prepared from the total lipid extract by transesterification using sodium methoxide in methanol (Applied Science Laboratories, Inc., State College, PA). Free sterols were precipitated from the lipid extract with digitonin (Carmack *et al.* 1976). The digitonides dissolved in dimethyl sulfoxide (DMSO) were placed in a boiling water bath for 1 h and the free sterols partitioned into hexane after cooling.

FAME were separated by gas–liquid chromatography using a Varian Aerograph 1400 gas chromatograph equipped with a 4 m × 2 mm stainless steel column packed with 15% diethylene glycol succinate (DEGS) on chromasorb P. Operating conditions were carrier gas flow, 40 mL/min; injector and detector temperatures, 270 and 290°C, respectively. The oven temperature was programmed from 150 to 250°C at 10°C/min. Sterols were separated using the latter gas chromatograph equipped with a 4 m × 2 mm glass column packed with 3% SE-30 on Chromasorb P. Operating conditions were carrier gas flow, 40 mL/min; oven, injector, and detector temperatures 250, 270, and 290°C, respectively.

Until rust fungi (Uredinales) were cultured axenically, spores were the only material from this group of organisms available for study. Much of the early analytical research with rust spores was concerned with the total oil and fatty acid content and more recently with sterols. Those studies showed that rust fungi have at least two chemical characteristics distinguishing them from most other fungi (Weete 1973, 1974). First, rust spore oil contains the unusual fatty acid *cis*-9,10-epoxyoctadecanoic acid which ranges in concentration from 4 to 78% of the total fatty acids, depending on the species. Second, the predominant fungal sterol ergosta-5,7,22-dienol (ergosterol) has not been detected in rust spore extracts. Instead, rust spores contain 24-ethyl or 24-ethylidine sterols that are often accompanied by low relative amounts of ergost-7-enol (fungisterol).

In the absence of definitive diagnostic characteristics, confirmation of rust fungi in culture is by inoculation of the host with spores or mycelium produced *in vitro* with subsequent development of characteristic disease symptoms and stages of the rust life cycle. It seems reasonable to expect that mycelium and possibly yeast-like forms of rust are qualitatively similar to the fungal spores of the same species with respect to the characteristic lipid composition, and that analysis of these lipids may be used as a relatively simple and rapid test to support the identification of rust fungi in culture. The lipid composition of vegetative and reproductive structures of the same species has been compared in relatively few fungi. Although there are some exceptions, generally the fatty acid and sterol (free sterols plus sterol esters) composition is qualitatively similar in spores and mycelium (Weete 1974). The fatty acid and sterol composition of *C. fusiforme* aeciospores and basidiospores collected from their natural hosts have been previously reported from this laboratory (Carmack *et al.* 1976; Weete and Kelley 1977). When it came to our attention that mycelial and possibly yeast-like forms of *C. fusiforme* were available, it seemed that this fungus would be suitable for determining whether spores and mycelium of this species contain the epoxy $C_{18}$ and characteristic sterols, and whether yeast-like organisms isolated during basidiospores germination may be *C. fusiforme*.

TABLE 1. Fatty acids of mycelial and yeast-like forms suspected of being *C. fusiforme*

| | Mycelium | | Yeast-like forms[a] | |
|---|---|---|---|---|
| Fatty acid | Orange | White | KB | KC |
| $C_{12}$ | 1.9 | 1.2 | — | — |
| $C_{14}$ | 0.3 | 3.0 | 1.2 | 0.8 |
| $C_{15}$ | 0.6 | 3.0 | — | Trace |
| $C_{16}$ | 18.7 | 24.0 | 18.9 | 10.5 |
| $C_{16:1}$ | — | — | 0.7 | 10.4 |
| $C_{18}$ | 4.1 | 4.0 | 5.6 | 2.0 |
| $C_{18:1}$ | 19.0 | 14.6 | 50.3 | 43.9 |
| $C_{18:2}$ | 30.7 | 26.0 | 14.1 | 14.1 |
| $C_{20}$ | — | — | — | — |
| $C_{18:3}$ | 13.2 | 12.7 | 0.8 | 14.2 |
| $C_{18}$ epoxy | 5.9 | 2.6 | — | — |
| $C_{24}$ | — | — | 4.1 | 1.5 |

[a]KB and KC are arbitrary designations for the yeast-like isolates.

The mycelium used in this study has been previously confirmed in culture as a rust fungus by the presence of yellow structures resembling the aecial sori containing mature aeciospores and by the production of disease symptoms typical of the fusiform rust disease after inoculation of the primary host *Quercus nigra* L. with spores from the laboratory culture (Hollis *et al.* 1972). Two separate mycelial isolates were analyzed, one white and the other faintly yellow–orange. Total lipid comprised 26 to 30% of the mycelial dry weight, which is high compared to most fungi but similar to the level in spores of *C. fusiforme* (Weete and Kelley 1977). The fatty acid composition was similar for the two mycelial isolates (Table 1), but quantitatively different from that of the aeciospores (Carmack *et al.* 1976) and basidiospores (Weete and Kelley 1977) of *C. fusiforme*. Epoxy $C_{18}$ was detected in both mycelial isolates, but in low relative proportions compared to that of the spores. The epoxy $C_{18}$ acid represented 2.9 and 5.9% of the white and orange mycelium, respectively, while it represents 49.4 and 40.7% of fatty acids in the aeciospores and basidiospores, respectively (Carmack *et al.* 1976; Weete and Kelley 1977).

Only the white mycelial isolate was analyzed by gas chromatography for sterols and was found to contain stigmast-7-enol (68%), ergost-7-enol (13%), and a $C_{29}$ diene which was probably stigmasta-5,7-dienol (20%). This composition was similar to that of *C. fusiforme* basidiospores (Weete and Kelley 1977) but different from that of aeciospores which contained mainly stigmasta-7,24(28)-dienol (98%) (Carmack *et al.* 1976). The similarity would be expected since the mycelium developed from germinating basidiospores. This similarity in sterol content, absence of ergosterol, and the presence of the epoxy $C_{18}$ acid support the identification of the mycelium as that of a rust fungus.

Unlike the closely related smut fungi (Ustilaginales) (Alexopoulos 1962), yeast-like forms of rust fungi are unknown. However, yeast-like budding has been observed by several investigators while attempting to germinate the spermatia of certain rust species (Scott and McLean 1969). In those cases, there was a question regarding whether the yeast-like budding was the rust or was due to a contaminating fungus. Several yeast-like colonies developed in culture during basidiospore germination of this study and were suspected of being *C. fusiforme*. Isolates of these yeast-like organisms were analyzed for evidence that would support their identification as the rust fungus. The yeast-like isolates contained ergosterol (about 98%) and none of the sterols typical of the *C. fusiforme* spores or mycelium. Also, the epoxy $C_{18}$ fatty acid was not detected in lipid extracts of the yeast-like isolates. Although not unequivocal, these data suggest that the yeast-like isolates are not a form of *C. fusiforme*.

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