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Role of *Laccaria laccata* in protecting primary roots of Douglas-fir from root rot

DAVID M. SYLVIA
Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA

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Summary The influence of *Laccaria laccata* on *Fusarium oxysporum*, and its ability to induce resistance to the pathogen in primary roots of Douglas-fir were investigated. Extracellular metabolites of *L. laccata* inhibited *F. oxysporum* hyphae, and delayed germination of microconidia and chlamydospores. *Laccaria laccata* or its cell-free metabolites also induced accumulation of osmiophilic materials in cortical cells of the primary root. The osmiophilic materials were primarily phenolic in nature. One-month-old primary roots, incubated with *L. laccata* or killed inoculum, were challenged with *F. oxysporum*. Rate of growth of the pathogen toward the root and intensity of surface colonization were not influenced by *L. laccata*, thus antibiosis was judged unimportant in root protection. However, cortical infection of the primary root by the pathogen was significantly less in seedlings affected by *L. laccata* than in controls. The frequency of hyphae was inversely proportional to the concentration of osmiophilic materials. Phenolics induced by *L. laccata* in the primary root are thus associated with resistance and may be the chemical basis for root protection.

Introduction

Root rot of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] by *Fusarium oxysporum* Schlect. emend. Snyd. & Hans. may be lethal if the primary roots of very young seedlings become infected[2]. The ectomycorrhizal fungus *Laccaria laccata* (Scop.: Fr.) can protect young seedlings of Douglas-fir by colonizing the primary roots shortly after germination[3,11]. Of the mechanisms for root protection proposed by Marx[10], *L. laccata* may protect roots by antibiosis, induced resistance, or alteration of rhizosphere microflora.

The objectives of this research were 1) to evaluate the potential for antibiosis of *L. laccata* to *F. oxysporum*, 2) to characterize the reaction of primary roots of Douglas-fir to *L. laccata*, and 3) to determine the significance of antibiosis and induced resistance in root protection. Details of these studies have been published elsewhere[12,13].

Materials and methods

Antibiosis

Two-wk-old cultures of *L. laccata* were comminuted for 5 sec with sterile water and dispensed to one side of petri plates containing Melin-Norkrans agar medium[9] plus thiamine and biotin at various glucose concentrations and pH. After 2-wk incubation at 21°C, plugs from the margin of cultures of *F. oxysporum* were placed 40 mm from the edge of *L. laccata* colonies and growth was observed for 1

month. Fractorial experiments were conducted involving pH (4 and 6), glucose concentration (3, 10 and 50 m*M*), and temperature (15, 21 and 27°C). Inhibition in each treatment was evaluated by dividing the distance that *F. oxysporum* grew toward *L. laccata* by the distance it grew toward an agar slurry on a control plate.

The activity of extracellular metabolites of *L laccata* were studied by growing the fungus in Melin-Norkrans liquid medium at 3 glucose levels (3, 10 and 50 m*M*). One-ml amounts of slurry from stock cultures were added to 125 ml Erlenmyer flasks containing 60 ml of a test medium and incubated 14 days at room temperature on a reciprocating shaker at 100 excursions/min. The contents of each flask were poured through filter paper, and the filtrate was sterilized by aspiration through 0.2 μm Nalgene membrane filters. Microconidia or chlamydospores of *F. oxysporum* were placed in the filtrate which was then added dropwise to sterile microscope slides and incubated in moist chambers. Spore germination was monitored periodically for 24 h.

Induced host response

Seedlings were grown in a wick culture system which allowed observation of primary roots and minimized damage at harvest[12]. Roots of intact seedlings grew gnotobiotically with *L. laccata* or stem-killed inoculum in 200 × 32 mm culture tubes while shoots were exposed to ambient conditions of the growth chamber. In a modification of the wick culture system, inoculum was separated from the root by a dialysis membrane (12,000–14,000 MWCO) to allow observation of effects of extracellular metabolites. After 6 wk seedlings were removed from the wick system and observed with light microscopy when fresh or after embedment in epoxy resin. Transverse sections of fresh roots were placed in water or a histochemical reagent. The following reagents were used: for phenolics, nitrous acid[4] or saturated alcoholic vanillin-concentrated HCl[7]; for lipids, Sudan black B[1]; for lignin, phloroglucinol[8]; for proteins, naphthol blue-black[6] or acid fuchsin[5]. Autofluorescence of fresh, unstained sections was observed with incident light fluorescence microscopy. Roots embedded in plastic were fixed in 1% glutaraldehyde plus 0.5% caffeine in 0.05 *M* sodium cacodylate buffer, postfixed with 2% osmium tetroxide, dehydrated in an acetone series, and embedded in Spurr's epoxy resin.

Root protection

Seedlings were grown in wick culture tubes with *L. laccata* or killed inoculum for 4 wk and then aseptically transferred to microscope slides coated with 2% water agar. Surface sterilized seed of Douglas-fir, internally infested with *F. oxysporum*, were placed on the agar near the root tips. Growth of hyphae toward roots was recorded daily, and after 10 days the distal 10 mm of the root was excised and prepared for light and scanning electron microscopy. Segments for scanning electron microscopy were fixed and dehydrated as described above, subjected to critical point drying, and coated with Au-Pd before viewing.

Colonization of the root surface by *F. oxysporum* was quantified from scanning electron micrographs made at three random locations along the root. A grid of dots 5 mm apart was placed on each micrograph, and the number of dots on hyphal images was expressed as a percentage of total dots over hyphae plus root surface. A cortical infection index was determined for three roots per treatment by viewing longitudinal sections through a net reticle. The number of intersections of the grid under which hyphae were seen was expressed as a proportion of the total number of intersections over cortical tissue. The relationship between osmiophilic compounds and fungal presence in the root cortex was quantified by determining, in conjunction with the assessment of internal infection, the absorbance of light by sections stained with osmium tetroxide.

Results and discussion

Antibiosis

Substantial inhibition of hyphal elongation of *F. oxysporum* in the presence of *L. laccata* occurred only on a medium incubated at 15°C containing 10 m*M* glucose at pH 4. Under this condition, hyphal length was reduced by 70% compared to the control. Antagonism was greater at 10 m*M* than 3 m*M* glucose, but significant interactions occurred among the factors. Extracellular metabolites of *L. laccata* delayed germination of *F. oxysporum* spores. After 11.5 and 20 h incubation in filtrate of *L. laccata*, germination of chlamydospores and microconidia, respectively, were reduced 64 and 55% as compared to controls.

Induced host response

Transverse sections of 10-day-old tissue of roots grown gnotobiotically with *L. laccata* contained darkly stained osmiophilic materials in cortical cells. Similar sections produced a positive reaction with phenolic reagents throughout the cortex. Control roots lacked osmiophilic materials in the cortex and there was little reaction with phenolic reagents in this tissue. Phenolics accumulated in the cortex of roots separated from *L. laccata* by a dialysis barrier, indicating that an extracellular fungal metabolite induced this response. Induced materials failed to react with reagents for lipid, lignin and protein, and did not autofluoresce.

Root protection

Prior colonization of root by *L. laccata* or exposure to its metabolites did not reduce either the rate at which *F. oxysporum* approached the root ($\bar{x}$ = 9mm/day) or the extent of surface colonization. *Fusarium oxysporum* colonized 18% of the surface of control roots compared to 43 and 25%, respectively, for roots exposed to *L. laccata* or its metabolites. Thus we judged antibiosis unimportant in root protection although *L. laccata* had been shown capable of inhibiting *F. oxysporum* in vitro.

Cortical invasion of roots by *F. oxysporum* was reduced significantly in seedlings that had been exposed to *L. laccata* or its metabolites. *Fusarium oxysporum* occurred over 25% of the surface of sections from control roots compared to 0 and 5%, respectively, for roots exposed to *L. laccata* or its metabolites. A highly significant, inverse relationship was found between hyphal frequency and accumulation of osmiophilic material in cortical cells. This induced host response may be the chemical basis for root protection.

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