Growth Medium for Dual Cultures of Loblolly Pine Callus and *Cronartium fusiforme*

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ABSTRACT

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A medium was developed for the simultaneous culture of loblolly pine callus and *Cronartium fusiforme* of basidiospore origin. Gresshoff and Doy medium 1 was modified by using different nitrogen concentrations, growth regulators, 2% glucose, adding 0.05% peptone, and 0.05% of an 80% ethanol-soluble fraction of yeast extract. This medium permitted good growth of both cultures without serious detriment to either organism during interactive studies. Light and temperature conditions suitable for survival and growth of both organisms are given.

Additional key words: fusiform rust.

In vitro growth of callus tissues of loblolly (*Pinus taeda* L.) and slash (*Pinus elliottii* var. *elliottii* Engelm.) pine was first achieved by Brown and Lawrence (3). Recently, the axenic culture of *Cronartium fusiforme* Hedgc. & Hunt ex Cumm. (*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Cumm.) Burds. et Snow.) also was attained (2,5,6). The literature contains few reports regarding the dual culture of these organisms. Hollis et al (6) found that cultures of *C. fusiforme* survived on his yeast extract-peptone amended medium, but slash pine callus on the same medium died within 6 wk. In contrast, Hare (5) cultured both *C. fusiforme* and slash pine callus on the medium of Hollis et al (6) for up to 2 mo. No intracellular fungal growth occurred.

The yeast extract required in a medium satisfactory for *C. fusiforme* is an impediment to dual culture studies since yeast extract causes rapid decline and eventual death of certain callus tissues (1). Preliminary experiments in this lab indicated that loblolly pine callus grown on the yeast extract and peptone medium of Hollis et al (6) did not survive or remain healthy for more than 2 wk. Thus, loblolly pine callus either may be more sensitive than the slash pine used by Hare (5), or more likely, callus health may be rated differently by various workers. This paper reports the development of a medium allowing the simultaneous culture of both *C. fusiforme* of basidiospore origin and healthy loblolly pine callus.

MATERIALS AND METHODS

Pathogen. Axenic cultures of *C. fusiforme* were obtained from basidiospores cast from excised telial columns as described by Amerson and Mott (2). The axenic colonies were initiated and grown on a modified Gresshoff and Doy medium 1(GD-1) with yeast extract (YE) and peptone, in the dark at 20–22 C (2,10). Telial columns were obtained from leaves of red oaks (*Quercus rubra* L.) inoculated with a mixture of aeciospores from 30 loblolly pine galls collected on the Coastal Plain of South Carolina and Georgia. Homogenates of mycelium in water were used to initiate new stock cultures by spreading the slurry on plates of GD-1, YE and peptone. The inoculum was available within 2–3 wk for use in subsequent experiments.

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Host tissue. Callus cultures of loblolly pine were initiated from segments of succulent stems obtained from greenhouse-grown 3-yr-old seedlings from seeds of field trees in Livingston Parish, LA. The stems were scrubbed with a brush and detergent, surface sterilized for 10 min with 0.8% sodium hypochlorite, rinsed for 30 sec in 70% ethanol, and finally rinsed three times (5–10 min each) in sterile distilled water. Stem sections (5–10 mm) were split longitudinally, placed cut side up on a modified Brown & Lawrence (B&L) medium minus calcium chloride (3) and cultured in continuous darkness at 20–22 C. The B&L medium was modified by lowering the 6-furfurylaminopurine (kinetin) concentration to 0.23 μM, the 2,4-dichlorophenoxyacetic acid (2,4-D) to 4.5 μM, and increasing the amount of agar to 1% (w/v). Callus used in the experiments was subcultured two to four times by transfers every 3–4 wk to B&L medium with calcium chloride.

Media. Several combinations of the nitrogen sources NH_4 + (NH_4NO_3) and NO_3^- (KNO_3) were examined in GD-1 medium. Among these were NH_4/NO_3 ratios of 20/40 mM (GD-1, 20/40) and 5/35 mM (GD-1, 5/35). These concentrations were chosen to approximate those found in the B&L medium and to provide concentrations that might produce susceptible host tissue. The source of the NH_4^+ ion was also changed in some experiments from $(NH_4)_2SO_4$ to NH_4NO_3 to reduce the sulfur concentration; this is hereafter referred to as GD-1, 5/35 (M). One growth factor regime tested consisted of kinetin at 0.23 μM and 2,4-D at 4.5 μM (noted as K, 2,4-D). Another was 2.2 μM 6-benzylaminopurine (BAP) and 2.7 μM α-naphthaleneacetic acid (NAA) (noted as BAP, NAA). Peptone was used at 0.05% or 0.1% (w/v). All media were adjusted to pH 5.5 with NaOH and HCl.

Two fractions of yeast extract were prepared by dissolving 50 g of yeast extract in 100-ml glass distilled water to which 400 ml of absolute ethanol was added. The mixture was stored at 4 C for 18 hr. The 80% ethanol-insoluble fraction (YE[I]) was removed by filtration and dried to a semisolid state by vacuum evaporation and air-drying at 35 C. The ethanol soluble fraction (YE[S]) was dried by the same process then used in media at 0.05 and 0.1% (w/v).

A chemically defined medium was prepared with GD-1 basal salts, vitamins and myoinositol, with amino acids and other compounds added at rates used previously by Hollis et al (6). Ornithine and oleic acid were not added to the medium, but 2.2 μM of BAP and 2.7 μM of NAA were added. The fungus growth was measured by taking diameter measurements of 10 colonies, using a dissecting microscope, every 5 days for 35 days.

RESULTS

Preliminary results with several modified media indicated that both loblolly pine callus and *C. fusiforme* (basidiospore form) grew for a limited time (4 wk) on a modified Gresshoff and Doy medium (Table 1). The best medium obtained, however, for the growth of both pine callus and *C. fusiforme* was medium 4 (Table 1). This medium consisted of the basal medium (GD-1) with inorganic salts, nitrogen at an ammonium-to-nitrate ratio of 5 mM to 35 mM, a carbon source, vitamins, BAP, NAA, peptone, and 0.05% of the 80% ethanol soluble fraction of yeast (Table 2). The fungus, however, did not grow well on any media when placed next to callus (8).

Growth of the callus. The growth and condition of loblolly pine callus on the media with lowered nitrogen concentrations (5/35 mM) were similar to the growth and condition on the standard medium (B&L) that contained a higher nitrogen concentration (20/40 mM) (Table 1, medium 3). Kinetin and 2,4-D gave slightly better results than BAP and NAA throughout the experiments (Table 1). The addition of YE(S) and peptone at 0.05 and 0.1% concentrations did not significantly inhibit callus growth (Table 1, media 4-7). This is an improvement over the browning and death of callus on media with YE(I) or whole yeast extract (7). Callus growth on the chemically defined medium was extremely poor; browning and death occurred by day 15 (Table 1, media 9).

Growth of the pathogen. The fungus did not grow without media amendments such as YE, peptone or amino acids (Table 1). *C. fusiforme* grew well on GD-1 media containing YE(S) with peptone or whole yeast extract with or without peptone. The growth on YE(S) + peptone was less than that on YE in the standard medium (Table 1, media 1), but the lower growth rate was acceptable (Table 1, media 4-8). When combined with peptone in the basal medium (GD-1), YE(S) supported good growth of the fungus (Table 1) whereas individually they did not support adequate growth. A YE(S) concentration of 0.1% and peptone did not significantly increase the growth over that on 0.05% YE(S) (Table 1). Growth of the fungus on media with the tested ammonium and nitrate concentrations was not noticeably different. BAP and NAA growth factors allowed good fungal growth, but kinetin and 2,4-D decreased growth at both concentrations of YE(S) and peptone (Table 1, media 4-7). The density or amount of mycelium was good on all media, except when grown on the chemically defined medium. Mycelium density or amount was rated subjectively. Radial growth on the chemically defined medium was also considerably less than that found on media with YE(S) or whole YE (Table 1, media 4-8).

DISCUSSION

The soluble fractions of yeast extract added to a basal medium allowed good growth of *C. fusiforme* and loblolly pine callus for at least 1 mo. The essential nutrients that whole yeast extract supplies to the fungus were still present in sufficient amounts in the soluble fraction. The removal from yeast extract of high-molecular-weight polysaccharides, insoluble in 80% ethanol, eliminates most of the substances that induce cellular stress in the pine callus (1).

The lower concentration of YE(S) is recommended as a precaution against inhibiting pine callus with any toxic substances remaining in the YE(S). The lower nitrogen concentrations are suggested for studies of host-pathogen interactions because high nitrogen concentrations may inhibit infection (9).

Growth factors were needed for the maintenance of pine callus. BAP and NAA, at the concentrations specified, appear to be a better choice than kinetin and 2,4-D since the latter two caused significant reduction in fungal growth on medium 6 (Table 1). The defined culture medium (Table 1, medium 9) did not support the growth of both organisms and appeared to need more refinement to allow callus and fungus growth.

The major benefit of the new medium (GD-1, 3/35, BAP, NAA, YE(S), and peptone) was the ability to grow an "obligate" fungal pathogen and host callus on the same medium without serious detriment to either for at least 1 mo without transfer. The problems

TABLE 1. Growth and condition of loblolly pine callus and *Cronartium fusiforme* on various media with amendments

	Callus				Fungus
	Day 15		Day 30		
Media[a]	Condition index[b]	Growth index[c]	Condition index	Growth index	Mean growth[d] (μm/5 days)
1. GD-1, 5/35(M), BAP, NAA	1.2	1.9	2.2	2.2	0
2. GD-1, 5/35(M), K, 2,4-D	1.2	1.1	1.2	1.1	0
3. B&L, 20/40, (M), K, 2,4-D	1.2	1.3	1.6	1.6	0
4. GD-1, 5/35(M), BAP, NAA, 0.05% YE(S) & P	1.2	1.7	2.1	2.1	1,020 x
5. GD-1, 5/35(M), BAP, NAA, 0.1% YE(S) & P	1.8	1.8	2.0	2.0	1,120 x
6. GD-1, 5/35(M), K, 2,4-D 0.05% YE(S) & P	1.4	1.9	2.3	1.8	860 yz
7. GD-1, 5/35(M), K, 2,4-D 0.1% YE(S) & P	1.1	1.6	1.7	1.7	970 xy
8. GD-1, 3/10, NAA.1 0.1% YE & P	3.0	3.0	4.0	4.0	1,390 w
9. GD-1, 3/10, BAP, NAA, Amino acids	4.0	4.0	4.0	4.0	750 z

[a] Abbreviations used in media types: GD-1 = Gresshoff and Doy medium 1; B&L = Brown and Lawrence; 3/10, 5/35 = the ratio of NH_4^+ to NO_3^- in mM; (M) indicates NH_4^+ is from NH_4NO_3 instead of $(NH_4)_2SO_4$; NAA.1 = 0.5 μM NAA; BAP = 2.2 μM BAP; NAA = 2.7 μM NAA; K = 0.23 μM kinetin; 2,4-D = 4.5 μM 2,4-D; 0.5% or 1.0% YE(S) & P = the 80% ETOH soluble fraction of yeast extract and total peptone; YE = whole yeast extract; amino acids = those of Hollis et al (6). All media adjusted with NaOH or HCl to a pH of 5.5.

[b] Callus condition index is the average of eight to 16 callus pieces, rated from 1 to 4, healthy to brown and necrotic.

[c] Growth index is the average of eight to 16 callus pieces rated from 1 to 4, good to poor.

[d] Average radial growth of mycelium derived from basidiospores of *Cronartium fusiforme* on a basal medium of GD-1, YE & P. Growth was measured every 5 days for 35 days. Means of fungal growth with common letter were not significantly different at $P = 0.05$ on Duncan's multiple range test.

TABLE 2. Composition of the dual culture medium for loblolly pine callus and *Cronartium fusiforme*[a]

Substance	mg/L	Substance	mg/L
KNO_3	1,515	$Na_2MoO_4 \cdot 2H_2O$	0.25
NH_4NO_3	400	$FeSO_4 \cdot 7H_2O$	27.8
$MgSO_4 \cdot 7H_2O$	250	Na_2EDTA	37.3
$MnSO_4 \cdot 7H_2O$	10	Thiamine HCl	1.0
$ZnSO_4 \cdot 7H_2O$	3	Nicotinic acid	0.1
$CuSO_4 \cdot 5H_2O$	0.25	Pyridoxin HCl	0.1
$CaCl_2 \cdot 2H_2O$	150	Inositol	10.0
KCl	300	Sucrose	20,000
KI	0.75	Agar	10,000
$CoCl_2 \cdot 6H_2O$	0.25	BAP	0.5
$NaH_2PO_4 \cdot H_2O$	90	NAA	0.5
$Na_2HPO_4 \cdot 7H_2O$	30	80% ETOH Sol. YE	500
H_3BO_3	3	Peptone	500

[a] Based on Gresshoff and Doy medium 1, media was adjusted with NaOH and HCl to pH of 5.5 prior to autoclaving.

of cell death and decline that were limiting factors in previous work (4–6) with dual culture were obviated. The ability to grow both the pathogen and the host tissue on the same medium makes possible studies of physical and chemical effects on pathogenesis that otherwise would be impossible.

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