Axenic Culture of the Mononucleate Stage of Cronartium ribicola

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ABSTRACT

Cultures of *Cronartium ribicola* were established on media containing (g/liter) calcium nitrate 0.5, magnesium sulfate 0.14, potassium phosphate (monobasic) 0.14, ammonium sulfate 0.025, ferric sulfate 0.014, manganese sulfate 0.0035, glucose 10.0, and Difco agar 10.0, plus any one of the following: (i) vitamin-free casein acid hydrolysate 10.0; (ii) yeast extract 1.0; (iii) Bacto peptone 1.0; (iv) Evan's peptone 1.0; (v) a combination of alanine 3.0, aspartic acid 3.0, and glutathione (reduced) 2.0; or (vi) as in v but substituting cystine 2.0 for glutathione. Excellent growth was obtained on the basal medium plus a combination of 1.0 yeast extract, 1.0 Evan's peptone, and 10.0 defatted Bovine serum albumin. Addition of $CaCO_3$ 10.0 increased growth, up to 300%, on all media. Inocula consisted of infected parts of tissue cultures of *Pinus monticola* or mycelium derived therefrom. Rust fungus transferred from five cultures reinfected pine tissue cultures and produced haustoria after 40 days in axenic culture (five subcultures). Subsequently, colonies from the five cultures were reestablished on artificial media. After 10 mo of continuous propagation, rust colonies from three cultures infected stems of germ-free *Pinus monticola* seedlings. All cultures intermittently produced immature spores. Homogenates of colonies from three cultures infected *Ribes nigrum* leaves and produced uredia and subsequently teliospores. Some cultures produced haustorialike structures.

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Additional key words: rust culture, saprophytic growth, defined medium, nutritional requirements, sporulation, host-parasite interactions, and host selectivity.

Isolates from at least five genera of rust fungi have been cultured to date, and have subsequently reinfected their respective hosts. In 1951, *Gymnosporangium juniperi-virginianae* Schw. was reported to have been cultured with a tissue culture system (8, 9, 19). Subsequently *Uromyces ari-tryphylli* (Schw.) Seeler and *Puccinia malvacearum* Bert. ex Mont. (10, 11) were also cultured in similar systems. Although these reports have not been subsequently verified by others, they are generally accepted. More recently, *Puccinia graminis* f. sp. *tritici* (Pers.) Erikss. and Henn. was cultured from uredospores (32, 33). These reports have since been verified by others (1, 2, 5, 12). Similarly, *Melampsora lini* (Pers.) Lev. has also been cultured from uredospores (29, 30) and has subsequently been verified (6). *Cronartium fusiforme* Hedgc. and Hunt ex Cumm. was recently reported in culture using techniques similar to those described herein (18). This report describes the axenic culture of the mononucleate stage of *Cronartium ribicola* J. C. Fisch. ex Rabenh.

MATERIALS AND METHODS.—*Inoculum.*—The original source of rust used in the studies reported here was about 25 rust cankers on western white pine (*Pinus monticola* Dougl.) from the St. Joe National Forest in northern Idaho. Mycelia were derived from these cankers via the infected-tissue culture technique previously reported (13). Inoculum (mycelium) used in establishing the axenic cultures was from either of two sources: (*a*) 10 mm^3 of rust-infected pine tissue cultures with aerial mycelium on one surface (13) or (*b*) 1 cm^2 pieces of dialysis membrane containing actively growing rust mycelium (14). No attempt was made to identify any given rust culture with individual rust cankers.

Media.—Two basal media were used to establish this rust in culture: (*a*) a medium containing a modification of Czapek's minerals, 0.1% yeast extract, 0.1% Evan's peptone, and 1.0% defatted Bovine serum albumin (BSA) (6), and (*b*) one previously developed for propagation of infected host tissue cultures (13). The composition of the second (tissue culture basal medium) was as follows (g/liter): calcium nitrate ($Ca(NO_3)_2 \cdot 4\ H_2O$) 0.5, magnesium sulfate ($MgSO_4 \cdot 3\ H_2O$) 0.14, potassium phosphate (KH_2PO_4) 0.14, ammonium sulfate [$(NH_4)_2SO_4$] 0.025, ferric sulfate [$Fe_2\ (SO_4)_3$] 0.014, manganese sulfate ($MnSO_4 \cdot H_2O$) 0.0035, 10.0 Bacto agar, and glucose 10.0 (final pH 5.5). Various additives were incorporated into the latter, including vitamin-free casein acid hydrolysate, yeast extract, Bacto peptone (Difco Laboratories, Detroit, Michigan); Evan's peptone (Evan's Medical Ltd., Liverpool); defatted (4) BSA fraction V (Miles Laboratories, Inc., Kanakee, Ill.); amino acids singly and in combination [based on a chromatographic analysis of rust mycelium (Harvey and Grasham, *unpublished*)]; and a combination of 10.0 BSA, 1.0 yeast extract, and 1.0 Evan's peptone. All media were poured into presterilized plastic petri plates, plates were 60 by 20 mm and contained approximately 10 ml each.

Incubation.—All materials were incubated under

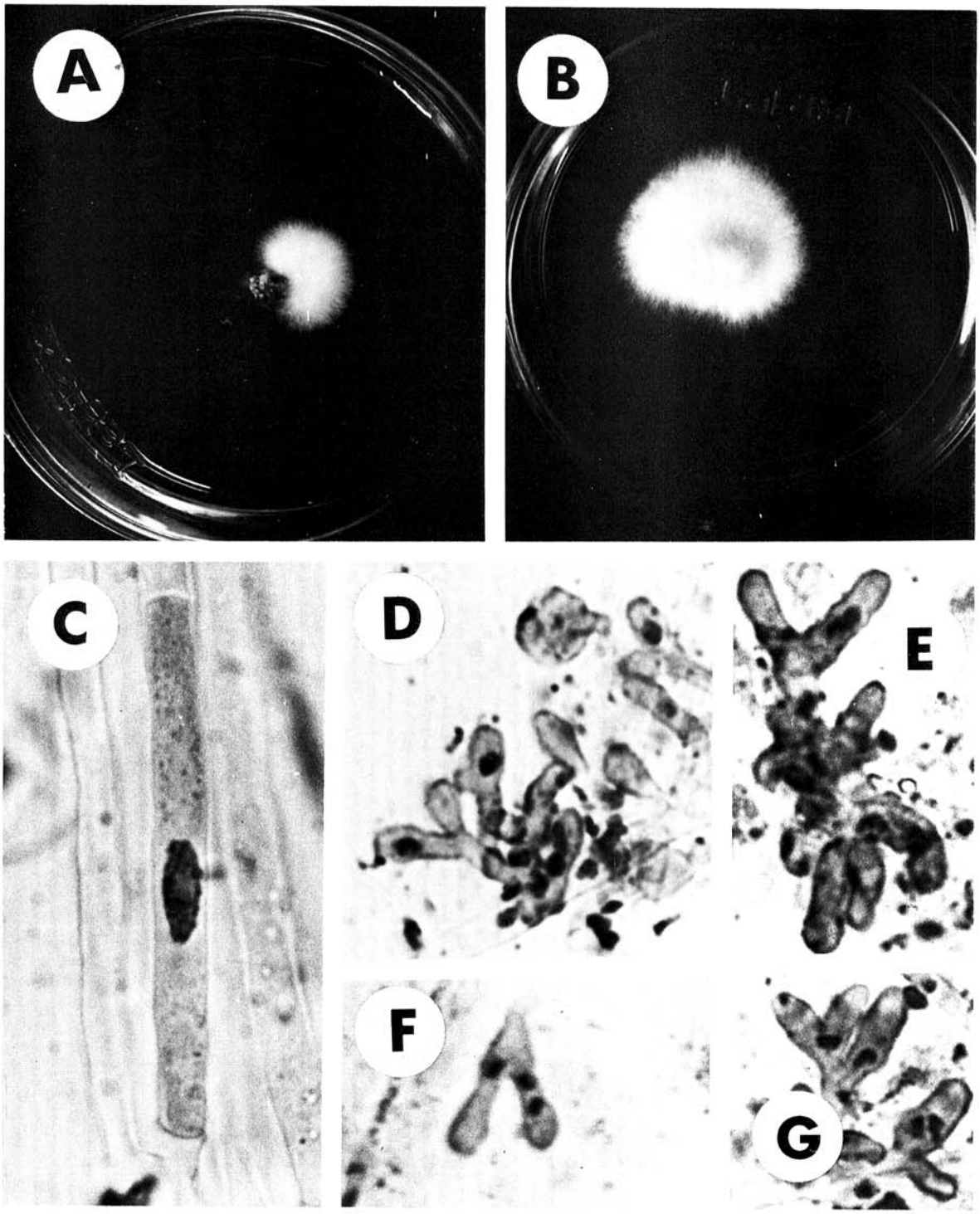

Fig. 1-(A to G). Cultural characteristics of *Cronartium ribicola*. **A)** 15-day-old culture of *C. ribicola* derived from inoculum source a, i.e., infected pine tissue culture (×2). **B)** 30-day-old culture derived from inoculum source b, i.e., mycelia from membrane culture (×2). Both cultures were grown on a tissue culture medium containing six mineral salts, 1% glucose, 1% BSA, 0.1% Evan's peptone, 0.1% yeast extract and 1% Difco agar. **C)** Mononucleate hyphal cell derived from cultures similar to the above (×375). Squash mount stained with giemsa. **D, E, F, G)** haustorialike structures produced in culture on dialysis membranes (×375). Prepared and stained as above.

standard conditions in a growth chamber environment identical to that in which the original inoculum was produced (13, 14), i.e., 24-h cyclic conditions with 16 h at 21 C, 4,300 lx of fluorescent light and 8 h at 5 C, no light.

In vitro infectivity tests.—Fifteen rust-free pine tissue cultures were inoculated with five selected cultures of the rust (three with each). The inoculum consisted of mycelia removed from the periphery of colonies (as they penetrated the medium beyond the membrane in inoculum source b). These mycelia were removed as 15 mm^3 of medium (0.1% yeast, 0.1% Evan's peptone, 1.0% BSA and the tissue culture basal medium as described above) with one surface covered with rust. These inoculum blocks were transferred onto the surface of rust-free tissue cultures, mycelium side down, and incubated under the standard conditions. Reisolation of the rust from the successful inoculations (40 days after inoculation) was achieved by seeding culture media with 10 mm^3 of the rust-infected tissue cultures. Only the medium containing 0.1% yeast, 0.1% Evan's peptone, and 1.0% defatted BSA in the tissue culture basal medium was used for reisolation. Five attempts were made from one of the three infected tissue cultures from each of the five selected rust cultures tested.

In vivo infectivity tests.—Inoculations of rust-free western white pine and *Ribes nigrum* L. plants were made according to the following procedures. Germ-free white pine seedlings were grown in the laboratory. One hundred pine seeds (current season) were sterilized by agitating 3 min in 100 ml of a 3% aqueous sodium hypochlorite solution to which 0.5 ml of Tween 20 (Nutritional Biochemicals, Cleveland, Ohio) had been added. Following this treatment, the seeds were rinsed in three 100-ml aliquots of sterile distilled water and placed (3 ea) in a 60 × 20-mm sterile plastic petri plate. Each plate contained 10 ml of an autoclaved [15 min, 1.41 kg-force/cm^2 (20 psi)] 10.0% solution of White's S-3 tissue culture medium (28), with no 2,4-D and with 10.0 g/liter sucrose and 10.0 g/liter Difco Bacto agar added to the final product. Seeds that germinated were transferred individually to 200-ml French square bottles containing 50 ml of the same substrate and capped with plastic food wrap. After 2 mo, epidermal tissues were surgically removed from a 2 × 50-mm strip of the lower stem from six seedlings. Whole colonies of individual 30-day-old rust cultures representing two cultures were introduced immediately, with the actively growing mycelium appressed to the wounds. Incubation was carried out under the standard conditions.

Isolated (growth chamber- or greenhouse-grown) *R. nigrum* plants were inoculated by atomizing homogenates of spore-producing rust colonies from each of the 12 cultures onto the abaxial leaf surfaces of two plants. Homogenates were prepared by blending 30-day-old rust colonies in 10 ml of distilled water for 45 sec (medium speed) in a Virtis model 30 homogenizer. One or two plants for each culture (1, 2) were atomized with the distilled water only. All were covered (individually) with plastic bags and incubated for 72 h under the standard conditions reported herein.

Measurements.—All growth measurements of this rust fungus were based on an average of 10 colonies, two each of the five isolates selected for testing. Data were taken as two right angle measurements of diam or as oven-dry weights of each colony. Unless otherwise specified, growth-rate measurements were made of colonies on the surfaces of dialysis membranes (14).

RESULTS.—*Media.*—The first axenic growth of this fungus was by three colonies from inoculum source *b*, on medium *a* (a modification of Czapek's minerals, 0.1% yeast extract, 0.1% Evan's peptone and 1.0% defatted BSA). Growth on this medium was slow; after 30 days, mycelia had penetrated the media about 1 mm. Each colony was then divided into three parts and transferred onto pine tissue cultured on the basal medium as described earlier plus 0.1% Evan's peptone, 0.1% yeast extract, and 1.0% defatted BSA. Within 30 days, the nine colonies had reached an average diam of 0.5 cm. On the basis of previous observations on the apparent effect of membranes on growth of this rust fungus (16), each of these nine cultures were then halved, and one-half was subcultured onto identical media and the other half onto sterilized dialysis membranes overlying the surface of this same medium. The result was that colonies 0.75 cm (range, 0.5-1.0) in diam developed on media without membranes and colonies 2.5 cm (2.25-3.0) in diam developed on the membranes after an additional 30 days. Thereafter, most subsequent culturing and results reported herein were obtained with dialysis membranes.

A total of 50 cultures from inoculum source *b* and 15 from inoculum source *a* were attempted on the superior medium. Of these, all 15 from inoculum source *a* (Fig. 1-A) and 29 of the 50 from inoculum source *b* (Fig. 1-B) yielded vegetative growth. From these 44 cultures, the 12 growing most rapidly were selected for subsequent investigations. No saltation was observed in these cultures.

TABLE 1. Average colony diam (cm) on dialysis membranes from 10 cultures of *Cronartium ribicola* 30 days after transfer onto culture media containing four different sources of organic nutrients[a]

Concn (g/liter)	Mean colony diam (and ranges)			
	Casein hydrolysate (vitamin-free)	Yeast extract	Bacto peptone	Evan's peptone
0.1	0.75 (0.5-1.0)	0.5 (0.25-1.0)	0.5 (0.25-1.0)	0.75 (0.5-1.0)
1.0	1.4 (0.80-1.5)	2.0 (1.25-2.5)	1.8 (1.5-2.25)	2.0 (1.75-2.25)
5.0	1.3 (1.0-1.75)	1.75 (1.25-2.0)	2.0 (1.5-2.25)	2.5 (2.0-3.0)
10.0	1.5 (1.0-2.0)	0.2 (0.0-0.5)	1.75 (1.5-2.0)	1.75 (1.5-2.0)
20.0	1.0 (0.5-1.5)	0.1 (0.0-0.4)	1.5 (1.25-2.0)	1.5 (1.25-2.0)

[a]Added to the tissue culture basal medium, which included six mineral salts (see text) plus 1.0% Bacto agar and 1.0% glucose.

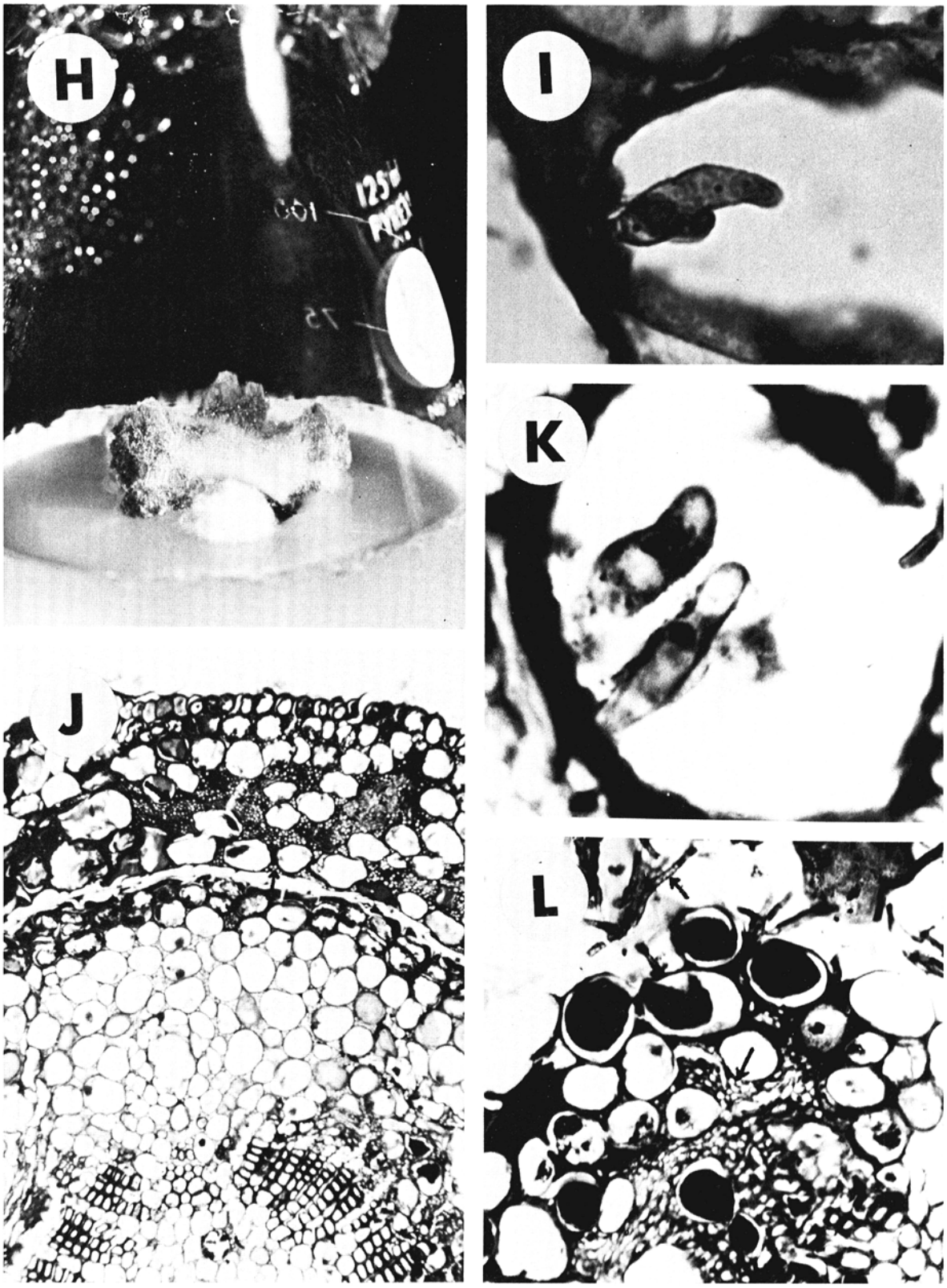

Fig. 2-(H to L). Axenic culture of pine tissue infected with *Cronartium ribicola*.**H)** Pine tissue culture 40 days after inoculation with mycelia from an axenic culture of *C. ribicola*. Note profuse development of aerial mycelia (×2). **I)** Haustoria formed in a rust-infected pine tissue culture (×375). Embedded in paraffin and stained with Flemming's quadruple stain. **J)** Cross-section of an 8-mo-old germ-free white pine seedling stem approximately 5 mo after inoculating with an axenic culture. Note the heavily ramified cortex region and frequent haustoria (×94). **K)** Haustoria formed in stem shown in J (×375). **L)** Cross-section of needle (cotyledon) from stem shown in J. Note the heavily ramified transfusion tissue (×188). J, K, and L prepared and stained as described above for I.

Physical environment.—There were two major environmental problems for axenic culture of this rust fungus; i.e., either wetting or desiccation of the fungus is frequently lethal. When transfers were made, great care was taken to prevent exposure to dry air or to noncolloidally bound (free) water. Excessive exposure to either resulted in failure. Further, any free water in media on which transfers were made resulted in poor growth.

Nutritional parameters.—Growth rates on the various complex sources of organic nutrients found to support this rust fungus in culture are shown in Table 1. In addition, the tissue culture basal medium with a combination of yeast extract and Evan's peptone (0.1% each) with and without 1.0% BSA, produced 2.5 cm (2.0-2.75) and 2.0 cm (1.5-2.25) colonies respectively after 30 days, and 3.0 cm (2.5-3.25) and 2.25 cm (2.0-2.5) respectively after 60 days. This growth pattern, characterized by gradual cessation of growth after 30 days, was evident on all these media. These cultures were not subculturable beyond 90 days.

This fungus did not grow on the standard tissue culture basal medium *b* (including glucose) with no additions, plus defatted BSA, plus any casein, yeast extract or peptone product treated with a cation exchange resin, or plus casein (in the absence of glucose) with or without the dialysis membrane. This prompted testing of filter-sterilized amino acids in conjunction with glucose and standard basal mineral salt medium. These tests demonstrated that this rust fungus would grow, for a minimum of five transfers (30-day intervals) on basal medium *b* to which only reduced glutathione or cystine (2.0 g/liter) had been added. Cysteine also supported limited growth, but even in the first subculture stage complete necrosis was common. Media which did not contain at least one of the sulfur-containing amino acids did not support growth. The best combination of defined nutrients resulted from addition of (g/liter) aspartic acid 3.0, alanine 3.0, glutathione (reduced) 2.0, glucose 10.0, and Difco agar 10.0 to the standard mineral medium *b*, previously developed for tissue cultures.

The rapid cessation of growth (after 30 days), an increasing opacity of the serum albumin component of appropriate media, and previous indications that *C. ribicola* [propagated by the membrane technique (14)], produced substantial quantities of a low molecular weight organic acid–possibly acetic acid or a derivative thereof (Harvey and Grasham, *unpublished*)–all prompted measurements of the pH of the media supporting rust colonies of this age. All media herein reported varied in pH from 5.4-5.8 at the time of inoculation. After 30 days, pH values were in the range of 3.8-4.5. Addition of $NaC_2H_3O_2$ 1.0 g/liter or $CaCO_3$ 10.0 g/liter increased growth approximately 15% on casein medium and up to 300% on all media, respectively. The latter was determined by dry weight.

In vitro infectivity tests.—All 15 inoculations made (three each of five cultures) produced tissue culture infections, with profuse aerial mycelium in the region of inoculation, within 40 days (Fig. 2-H). One infection from each of the five cultures was examined histologically 40 days after inoculation. Haustoria similar to those observed previously in host tissue cultures (17) were found in each infection (Fig. 2-I).

All 25 attempts to reisolate this rust fungus from infected tissue cultures (five each from the five cultures tested for pathogenicity) were successful. Colonies formed within 3 wk after they were transferred to artificial media.

In vivo infectivity tests.—Four of the six attempts to inoculate germ-free pine seedlings resulted in signs of infection. These signs included (i) swelling (up to twice normal size) and splitting of the stem in the region of the wound, (ii) appearance of aerial mycelium on the surface of the stem at points remote from the inoculation site, and (iii) yellowing of needle tissues, beginning at the base and proceeding toward the tip. Two seedlings representing potential infections, one from each of the two isolates, were examined histologically. Large quantities of mycelia (Fig. 2-J) and haustoria (Fig. 2-K) were distributed throughout the length and breadth of the stems. Invasion was greatest however, near the site of the inoculations and was coned within the cortical region (Fig. 2-J). Needle tissues were also heavily invaded (Fig. 2-L). In this instance the mycelia were coned in the transfusion tissue of the vascular bundle, but mycelia were evident throughout the needle as they were in the stems (Fig. 2-L).

Although attempts at inoculation of *Ribes* plants have been made with all our cultures, only three have thus far produced successful infections. In these, leaf spots were evident 12-20 days after inoculation. In all inoculations, uredia (Fig. 3-O, P) and telia were formed. Infectivity of the binucleate uredospores produced by these infections was confirmed by reinoculation. The technique substituted spores for colony homogenates, but was otherwise identical to that used for the axenic cultures. Noninoculated control plants showed no signs of infection.

Morphology.—All cultures produced only mononucleate mycelia (Fig. 1-C). The base of each culture consisted of a pale-yellow to gray mat covered with white aerial mycelium (Fig. 1-A, B). Overall colony morphology and pigmentation were somewhat different on the basal medium containing yeast extract as an additive. Here the mycelium was generally less dense and at high yeast extract concns (1.0 and 2.0%), pigmentation was dark-brown to black and leached from the fungus stroma into the medium.

Early in their history, our cultures formed few, if any, spores. However, after 3-4 mo, three of them regularly formed spores. These spores appeared to be immature and, within individual colonies, occurred at several stages of maturity. Although spore production has been erratic, all 12 cultures have been observed to sporulate. Typically, these spores are borne on sporogenous hyphal tips (Fig. 3-N), resemble immature aecia or uredia (Fig. 3-M, N), and occur within 0.5 cm of the growing edge of a colony. We have not been able to ascertain the nuclear condition, as yet, because of difficulties encountered in staining. No pycniospores were observed; however, pycnial-like fluid (containing no spores) has been observed in several colonies. After approximately 10 mo, one culture formed heavy deposits of yellow-orange pigment throughout its colonies, and has continued to produce this pigment. Pigment production was not dependent on spore formation.

As was true of spore production, after extended periods

of continuous propagation (approximately 12 mo), most of our rust cultures produced haustorialike structures. These occur in clumps (Fig. 1-D, E, G) and represent the only structure we have clearly determined to contain more than one nucleus. In one instance (Fig. 1-F), three nuclei occurred within a single structure.

DISCUSSION.—The choice of method for culturing this rust fungus was dictated by a desire to avoid the difficulties encountered by others in attempting to deal directly with germinating spores (22, 24, 31). We wished also to reprogram the parasite for saprophytic growth by partially isolating it from a highly-specialized to a less-specialized controllable environment (13, 14). The system we used, a modification of the general approach developed by Hotson and Cutter (19) gradually altered the environment from the in vivo circumstance to

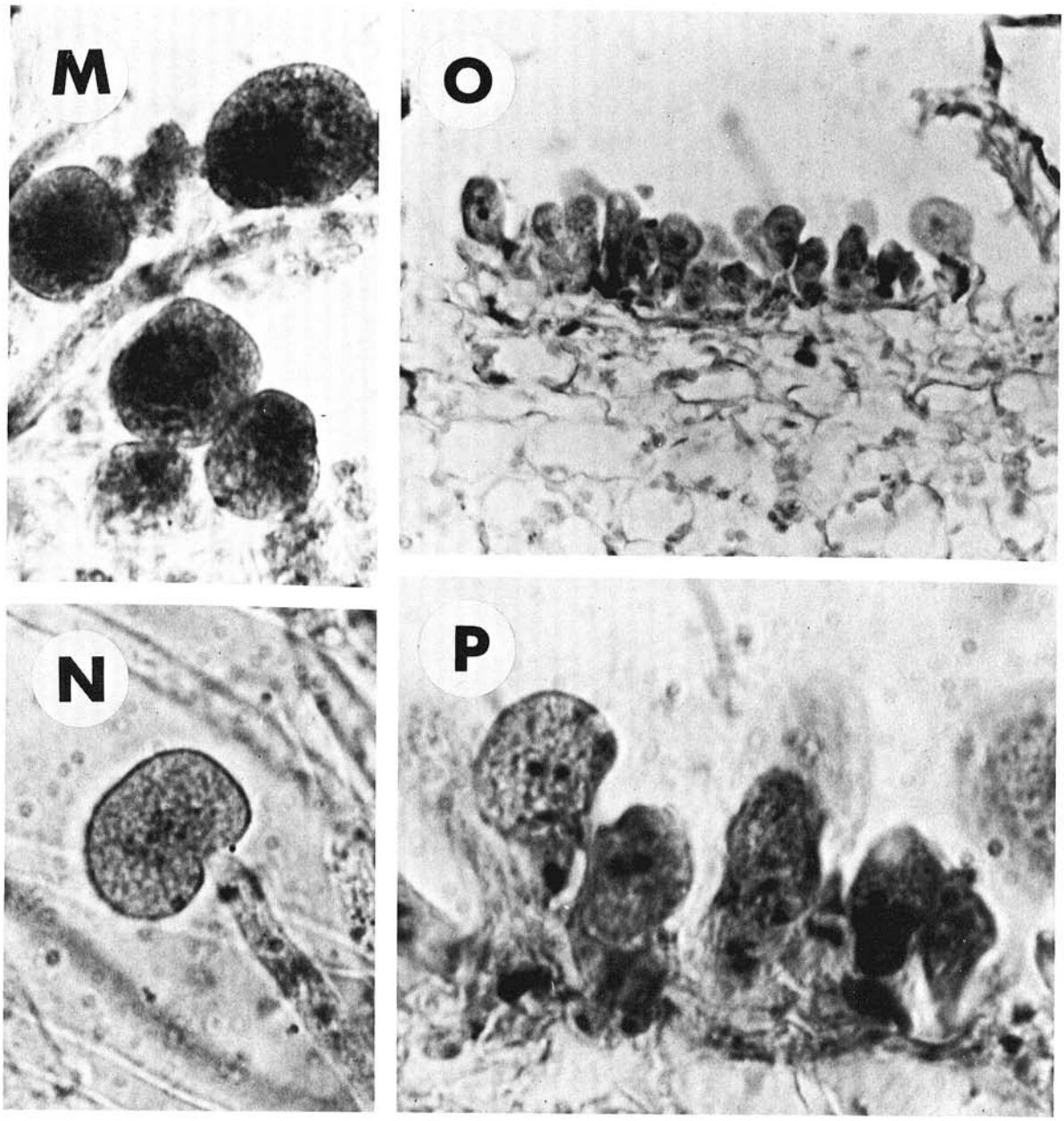

Fig. 3-(M to P). Sporulation of *Cronartium ribicola* on leaves of greenhouse- or growth chamber-grown *Ribes nigrum*. **M)** Cluster of spores typical of those produced by all isolates (×846). **N)** Same as in M, but spores not yet released from sporogenous hypha (×846). M and N are squash mounts stained with haematoxylin. **O)** Results of 16-day-old inoculation of *Ribes nigrum* leaves with homogenates of cultures producing spores as shown in M and N. Embedded in paraffin and stained with haematoxylin (×188). **P)** Spores from a pustule as shown above. Note nuclear condition (×846). Prepared and stained as described above for O.

controlled in vitro conditions more amenable to study of the nutrition and specialization of this parasite.

Of particular note in these cultures was the stability of their colony morphology and the fact that their mycelia were mononucleate. In contrast, saltation (26) and both mono- and binucleate mycelia have been obtained with other rust cultures (9, 22, 23). The haustorialike structures formed by our cultures strongly resembled haustorial types previously observed in infected pine tissue cultures (17). However, because these represent the only multinucleate structures we have observed, plausibly they could be aborted or immature fusion cells.

As reported for other axenic cultures of rust fungi (26, 33), staling was common with *C. ribicola* on all media, particularly when large amounts of mycelium were produced. This suggested a buildup of self-toxic metabolites within the culture media. Since buffering the media with $NaC_2H_3O_2$ or $CaCO_3$ alleviated this problem, acid production by the cultures was apparently responsible.

Most organic amendments thus far reported to support or enhance growth of rust fungi in axenic culture appear to act primarily as sources of nutrients. The effect of BSA on cultures of *C. ribicola* was additive, and occurred only in the presence of other sources of carbon and amino acid nitrogen, i.e., glucose and casein hydrolysate. Therefore, the benefit provided by this substance appears to be a non-nutritional phenomenon. Since BSA is a polycation which can specifically bind acetic acid (3), its effect may simply reflect neutralization of acid production. Because polycations are also known to influence gene expression (27), however, BSA or cationic subunits derived from the BSA might be active in this manner.

Although infection of *Ribes* leaves by rust fungus cultures cannot be ascribed to the spores alone since mycelial fragments were also included in the inoculum, the failure of some inoculations may have resulted from the failure of the spores produced in the artificial environment to consistently mature normally.

The nutrition of obligate parasites in various cultural systems is now sufficiently well understood to permit some interesting speculations. Cutter (9) suggested the possibility that the host range of *Gymnosporangium* may have been broadened by axenic culture. Furthermore, Peterson (25) reported an instance wherein *Cronartium* mycelia were observed parasitizing gymnosperm and angiosperm tissues simultaneously. Our own work has shown the ability of *C. ribicola* to parasitize nonhost tissue cultures (15), and the capability of the nutrients derived from the nonhost tissues to support growth (16). Chief among the nutritional requirements reported thus far for axenic rust cultures has been that for an external source of mineral nutrients, organic carbon, and nitrogen in the form of amino acids (7, 18, 21). Specific requirements for sulfur, amino acids, and/or aspartic acid have also been reported (7, 12, 20, 21). The requirement for an external source of mineral nutrients, carbon (in the form of glucose) and a sulfur-containing amino acid was noted in the present study. The nutritional requirements for rust fungi, therefore, could be potentially satisfied by many substances that are not restricted to the customary environment of the obligate parasite nor to specific plant genera, species, or cultivars. Thus, the determinant of the obligate nature and host selectivity of rust fungi seems not to be nutrition as such. We believe that such a determinant is more likely to be found in the parasite's mode of access to plant tissues (penetration and establishment), and in the ability of those tissues to tolerate or respond to the presence of specific metabolites derived from the parasite.

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