Single Genotype Axenic Cultures of *Cronartium quercuum* f. sp. *fusiforme*

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ABSTRACT

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Single genotype axenic cultures of *Cronartium quercuum* f. sp. *fusiforme* were isolated from individually cultured haploid basidiospores. Successful single spore initiations were obtained only on Harvey and Grasham (4) basal medium amended with 0.1 g/L $CaCO_3$, 1 g/L each of yeast extract and peptone, and 10 g/L of bovine serum albumin (HGYP + BSA). Nurse culture techniques were necessary for significant numbers of successful colony initiations. Nurse cultures were produced by casting basidiospores from a group of five clustered telial columns onto cellulose nitrate-acetate membranes that covered four test media, including HGYP + BSA. Basidiospores germinated and hyphae grew on these membranes for 2 wk but did not penetrate through to the underlying media. After 2 wk, the membrane was removed from the dishes to yield the conditioned nurse media. Four to 6 days after membrane removal, single basidiospores were inoculated onto the nurse media where overlying nurse spores had previously been cast. Single basidiospore colonies routinely developed in approximately 2 mo on HGYP + BSA nurse medium. This study is believed to be the first report of a rust fungus axenically cultured repeatedly from single spores.

Additional keyword: fusiform rust.

Theoretically, single genotype fungal colonies can be isolated from both single spores and hyphal tips (fragments). Both methods have been demonstrated with the fusiform rust fungus, *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Hedgc. & N. Hunt) Burdsall & G. Snow (5). This report will be limited to isolations from spores.

Amerson et al (1) described the influence of inoculum density on the frequency of colony initiation for *C. q. fusiforme*. They showed that colony initiation from each multibasidiospore location within a dish was enhanced as the basidiospore concentration increased per location or as the number of locations increased in a single dish. This result suggested that single spore isolates would be extremely difficult to obtain, but the apparent synergism among the spores gave promise of single spore isolation by using a nurse culture.

In a continuation of the work by Amerson et al (1), Frampton (3) obtained one basidiospore isolate of *C. q. fusiforme*. This spore developed into a hyphal colony on GDYP medium (1) by placing a high concentration of nurse spores adjacent to the single spore.

Attempts to repeat Frampton's procedure and a variety of other nurse culture procedures undertaken in the early phases of this current work failed on GDYP media containing either sucrose or glucose (5). In this paper, a detailed technique for growing a single genotype colony directly from a single basidiospore of *C. q. fusiforme* is reported, and the importance of nurse culture and nutrient medium is demonstrated.

MATERIALS AND METHODS

Aeciospores of *C. q. fusiforme* collected from two fusiform rust gall lines (VC-6 and 2-74) on loblolly pine were supplied by U.S. Forest Service personnel in Athens, GA. Red oak (*Quercus rubra* L.) seedlings were inoculated with these aeciospores according to USFS procedures (6) briefly summarized here. Five to 10 mg of vacuum-desiccated aeciospores were rehydrated for 24 h in a humid glass petri dish at 5 C. Hydrated spores were suspended in 30 ml of distilled water and sprayed onto the underside of succulent leaves of 30-day-old oak seedlings. The inoculated oak seedlings were incubated at 20–25 C in a dark, humid chamber for 24 h and then transferred to a shaded air-conditioned greenhouse chamber (20–25 C) for 15–20 days to permit telial column formation.

To generate nurse media for the two experiments described in this paper, the bases of individually excised telial columns (2), in groups of five, were embedded in 1.5% water agar poured into a petri dish cover (1,2). These covers were used to reassemble petri dishes, suspending the telial columns above culture media overlaid with 70-mm-diameter, 0.45-μm pore size cellulose nitrate-acetate membranes (Micron Separations Inc., Westboro, MA). The telial cultures were incubated in darkness at 21–25 C to permit spore casting. The spent columns were removed after 48 h. Basidiospores of unknown number cast onto the membranes were incubated and germinated in darkness at 21–25 C for 2 wk to yield small hyphal masses. Membranes then were removed from the dishes to yield the nurse medium. At the time of membrane removal, the positions of the overlying nurse sites on the membrane were marked on the petri dish covers and bottoms. Four to 6 days after membrane removal, single basidiospores in an aqueous inoculum were inoculated onto these marked sites.

Preparation of the aqueous inoculum containing basidiospores for use in single spore experiments followed Method II procedures of Amerson et al (1). Whole oak leaves bearing telial columns were attached with 1.5% water agar to the cover of a 150-mm-diameter petri dish. These leaves were suspended over pH 2.2 sterile glass-distilled water covering the dish bottom. They were maintained in darkness at 21–25 C for 36–48 h, during which time basidiospores were cast onto the pH 2.2 water, which inhibited their germination. Basidiospores were collected from the water by Büchner filtration concentrating them onto a sterile Millipore filter (5-μm pore size). Basidiospores on the filter were washed with 30 ml of sterile glass-distilled water, pH 5.5, and suspended in 1–5 ml of pH 5.5 sterile glass-distilled water. Spore concentrations in this initial dilution were determined with a hemacytometer. Further dilutions in pH 5.5 sterile glass-distilled

TABLE 1. Influence of four media and nurse culture[a] on the initiation of hyphal colonies from basidiospores (gall line VC-6) of *Cronartium quercuum* f. sp. *fusiforme* in diluted inoculum

	Media							
	HGYP + BSA		HGYP		GDYPS + BSA		GDYPS	
Treatments	1 sp.[b]	2+ sp.[b]	1 sp.	2+ sp.	1 sp.	2+ sp.	1 sp.	2+ sp.
1. Nurse medium + single spore	8/59/14[c]	13/79/16	0/68/0	0/73/0	0/42/0	5/72/7	0/43/0	0/88/0
2. No nurse medium + single spore	0/63/0	0/83/0	...	...	...	...	...	...

[a]Nurse medium controls without single spore inoculations produced no colonies from 60 nurse sites on HGYP + BSA and 40 nurse sites on GDYPS + BSA.
[b]Values for one spore inoculations (1 sp.) represent single spores physically separated from other spores. Values for 2+ spore inoculations (2+ sp.) represent spore clumps having two or more spores in close physical proximity.
[c]Number of successful initiates/number tested/percent success.

TABLE 2. Colony initiation from basidiospores (gall line 2-74) of *Cronartium quercuum* f. sp. *fusiforme* as influenced by the number of nurse sites[a] per dish on HGYP + BSA medium

Number of nurse sites[b]	Number of single spores[c]	Success rate[d]
0	69	1
20	49	31
21	83	28
22	87	18
23	56	20
24	35	40
25	72	26
26	101	20
30	132	21

[a]Nurse medium control without single spore inoculations produced no colonies from 150 nurse sites.
[b]Number of nurse sites in dishes inoculated with single basidiospores.
[c]Number of single basidiospores tested for a given number of nurse sites per dish.
[d]Percentage of single basidiospores (rounded off) making colonies.

water were made to achieve an average concentration of one basidiospore per microliter. All diluted inocula were slowly stirred with a magnetic stirrer and were chilled by an ice bath to prevent spore clumping and early germination.

The inocula, on average, contained one basidiospore per microliter, but any single microliter could contain no spores or several spores. To help insure that spores were present on the inoculated media, 2 μl of the inoculum suspension was placed at each inoculation site on the culture medium. When nurse medium was used, inoculations were placed directly on, or closely adjacent to, previously marked nurse sites. Within 2 days of inoculation, but subsequent to germination, the inoculated sites were microscopically examined to identify with certainty those sites containing single spores or multiple spores with sufficient physical separation to permit single spore observation and isolation. Subsequent to inoculation, cultures were maintained in darkness at 21–25 C and examined biweekly to monitor growth and development.

Experiment I was conducted with four different media: HGYP = Harvey and Grasham medium (4) amended with 0.1 g/L of $CaCO_3$ and 1 g/L each of yeast extract (Y) and peptone (P); HGYP + BSA = HGYP plus 10 g/L of bovine serum albumin (BSA); GDYPS = modified Gresshoff and Doy 1 medium with 1 g/L each of yeast extract and peptone and with 2% sucrose (S) substituted for glucose (1); GDYPS + BSA = GDYPS plus 10 g/L of BSA. All culture media were autoclaved at 121 C for 17 min at 15 psi and solidified with 1% Bacto agar (Difco, Detroit, MI). BSA was filter-sterilized and added into the cooled media immediately before the media were poured into 100-mm petri dishes. Basidiospores for this experiment were obtained from single gall line VC-6. Nurse culture media were prepared as previously described for all four of the above media. Ten nurse sites were established in a circle approximately 2.5 cm from the edge of each nurse dish. Five dishes of each nurse culture media were inoculated with 10 2-μl inoculum drops containing an average of one spore per microliter (treatment 1). Inoculation was directly on or adjacent to one of the 10 nurse sites in each dish to yield a total of 50 inoculation sites. Similarly, five dishes of HGYP + BSA, without the aid of nurse cultures, were inoculated with 15 2-μl drops of the above inoculum to constitute treatment 2. Ideally each nurse site would contain two physically separated spores, but this was not always the case. Nurse medium control dishes, not inoculated with any single spores, prepared with HGYP + BSA and GDYPS + BSA were included to determine if hyphae of nurse site origin could grow though the cellulose nitrate-acetate membrane and establish colonies that might be falsely identified as being of single spore origin. Six dishes of HGYP + BSA and four of GDYPS + BSA containing 10 nurse sites each were included in the experiment, and these were designated as the nurse media control group.

Experiment II was conducted to determine if the number of nurse sites over the range of 20–30 per plate significantly influenced single spore colony initiation. A no-nurse treatment also was included to reexamine treatment 2 in experiment I. Two to five dishes of HGYP + BSA containing 0, 20, 21, 22, 23, 24, 25, 26, or 30 nurse sites per dish were prepared using nurse spores from mixed gall line 2-74. Dishes were each inoculated with 20–30 2-μl inoculum drops containing an average of one spore per microliter. Nurse medium controls (five HGYP + BSA plates with 30 nurse sites each) without any single spore inoculations were included as in experiment I to further determine if nurse hyphae could penetrate the cellulose nitrate-acetate membranes.

RESULTS

Of the four media tested, only HGYP + BSA medium supported colony initiation from single spores. The success rate averaged 14% for that medium (Table 1). The suitability of HGYP + BSA medium is further demonstrated where initiation rates, dependent on specific nurse treatments, routinely equaled or exceeded 18% (Table 2). The use of nurse culture strongly promoted the initiation of single spore colonies in conjunction with HGYP + BSA medium, although nurse culture is not absolutely required (Tables 1 and 2). With basidiospores from gall line VC-6, eight of 59 (14%) single spores cultured on nurse medium HGYP + BSA produced colonies, whereas none of the 63 single spores cultured onto HGYP + BSA medium without nurse culture produced isolates (Table 1). In Table 2, a similar number of single spores (69) from gall line 2-74 produced only one single spore isolate on HGYP + BSA medium without nurse culture, whereas HGYP + BSA nurse treatments with 20–30 nurse sites per dish produced colonies from 18 to 40% of the single spores tested. Although the practical necessity of nurse culture for single spore colony

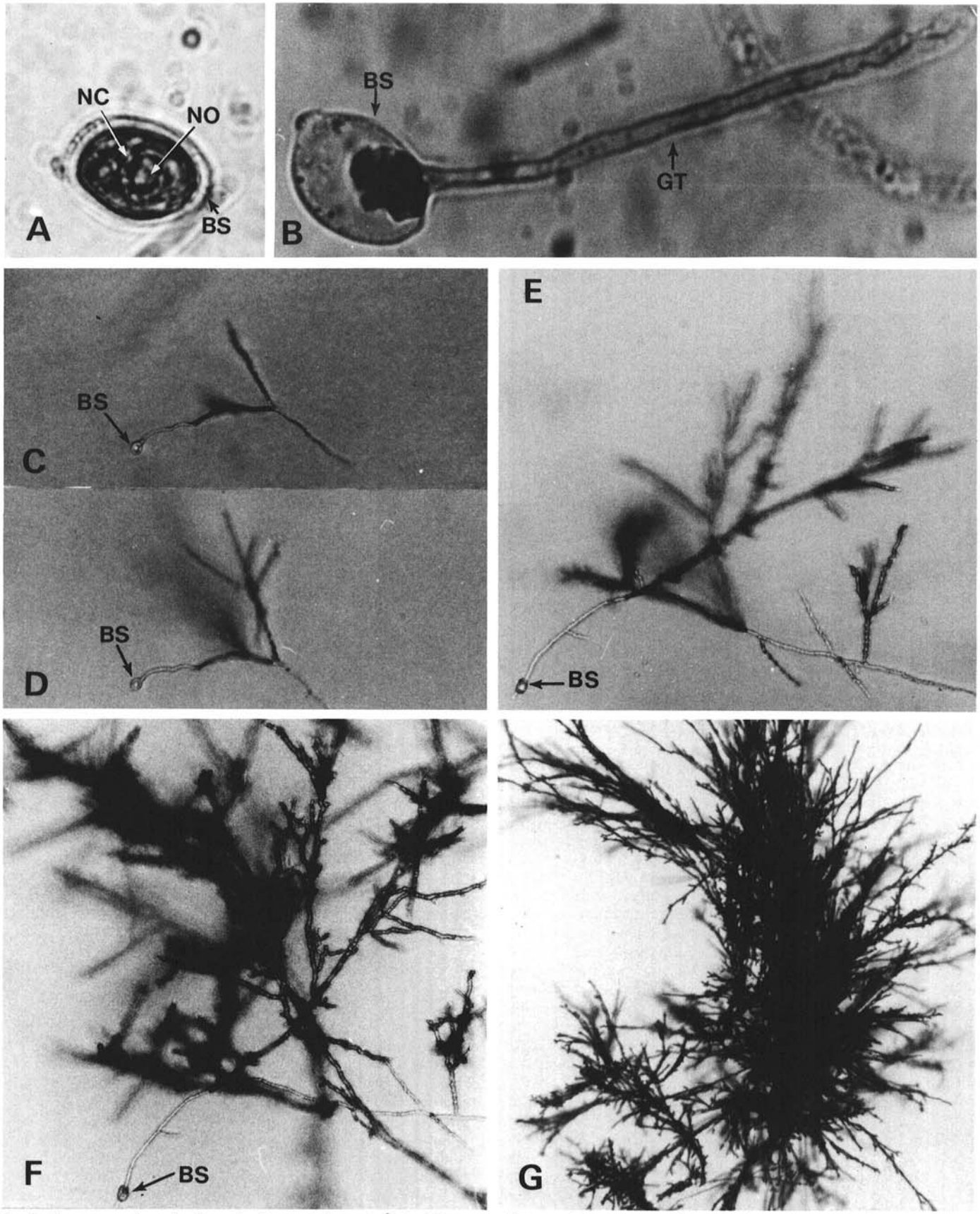

Fig. 1. The progression of colony development from single basidiospores of *Cronartium quercuum* f. sp. *fusiforme* on HGYP + BSA medium. **A,** Uninucleate basidiospore of *C. q. fusiforme* (2,000×). **B,** Basidiospore of *C. q. fusiforme* less than 24 h after germination (2,000×). **C,** Germinated single basidiospore of *C. q. fusiforme* with its first branch 1 wk after inoculation on culture medium (200×). **D** and **E,** Germinated single basidiospores of *C. q. fusiforme* with second and third order branching after 3 wk (200×). **F,** Highly branched culture formed from a single basidiospore of *C. q. fusiforme* (200×). **G,** Colony produced from a single basidiospore of *C. q. fusiforme* after 2 mo (80×). BS = basidiospore; GT = germ tube; NC = nucleus; NO = nucleolus.

initiations was demonstrated, increasing the number of nurse sites across the range of 20–30 sites per dish (Table 2) did not affect single spore colony production. No strong correlation existed between the number of single spore colony initiations and the quantity of nurse sites per dish ($R = -0.28$, $P = 0.4988$, $\alpha = 0.05$).

Nurse medium control treatments in both experiments I and II failed to produce any isolates from 250 opportunities (footnotes, Tables 1 and 2). This demonstrated that nurse cultures during the 2-wk incubation did not penetrate the cellulose nitrate-acetate membranes and colonize the medium. This lack of colonization in nurse controls, coupled with direct observations on single spore inoculations with and without nurse culture, assures that colonies (except 2+ spores inoculum) were of single spore origin. The typical pattern of colony establishment from a single spore is shown in Figure 1. A single basidiospore (Fig. 1A) typically begins to germinate on nutrient media within 24 h of inoculation (Fig. 1B). Branching routinely is observed in week 2 of development (Fig. 1C). After a 3- to 4-wk period, secondary, tertiary, or even higher orders of branching are formed (Fig. 1D–F). By the end of a 2-mo period, small white hyphal colonies reaching a size 1 mm in diameter (Fig. 1G) can be subcultured onto fresh medium. Unsuccessful basidiospores typically germinate on the nutrient medium, but display little or no branching. More rarely, highly branched, partially developed colonies may unexplainably cease development.

DISCUSSION

In producing hyphal isolates from single spores, both nurse culture and medium composition were shown to be important. Fewer than 1% of the single spores tested on a suitable medium (HGYP + BSA) without nurse sites yielded colonies, whereas 14–40% of the single spores on nurse medium HGYP + BSA yielded colonies (Tables 1 and 2). From these experiments, it is impossible to determine whether nurse cultures favorably condition the medium by adding some component(s) or by subtracting some component(s). However, by placing nurse cultures on a membrane that was not penetrated by the fungus, mobility of the amended or amending component(s) was demonstrated.

Amerson et al (1) demonstrated beneficial nurse culture interactions within and among multibasidiospore inoculations, and these facilitated the production of multigenotype colonies from multispore inocula placed directly on the medium. The use of nurse spores and hyphae on membranes removed 4–6 days before the placement of single spores directly on the medium now demonstrates that nurse culture benefits in the medium are residual and retained for at least 4–6 days in the absence of the nurse fungus. However, from these current experiments, we do not know if the nurse culture effect persists past 6 days.

With *C. q. fusiforme,* successful initiation of multigenotype colonies increased as the number of inoculum sites or the number of basidiospores within an inoculum site increased within a dish, showing a positive correlation between spore quantity and colony initiation (1). The same relationship might be expected for nurse culture sites and corresponding single basidiospore inoculations, but no such relationship has yet been shown. Because basidiospore nurse sites in the current experiments resulted from telial column casts that potentially contain different numbers of spores per cast, no data exist on the influence of spore numbers per site. Such studies are needed and should be conducted with quantified aqueous inoculum.

In experiment II, dishes with nurse sites over the range of 20–30 sites did not differ in their ability to aid single spore colony production, yet all were much better than the 1% value for the no-nurse control. If nurse culture alteration of the medium remains localized, quantitative aspects related to the number of sites may be unimportant. However, multigenotype data (1) suggest that nurse effects are not highly localized and that quantitative effects should be present. Further work on the topic of localized vs. widespread nurse benefits is needed.

Although nurse culture methods have been used to obtain hyphal isolates from low but unspecified numbers of rust urediniospores, presumably *Puccinia* (7), the present report, excluding the single isolate of Frampton (3), constitutes the first case where documented single spore initiation of a rust colony has been obtained. The use of the haploid colonies in studying rust genetics is potentially great, but the application remains to be shown.

Frampton (3) found that BSA aided the growth of developing multigenotype colonies derived from many spores. Similarly, BSA in our study is an important medium component for culture initiations beginning with single basidiospores or very small numbers of spores. Neither HGYP nor GDYPS without BSA supported colony initiation from spores, but HGYP appears to be a better basal medium than GDYP for production of cultures from single spores, given the 14% success rate of HGYP + BSA and the 0% rate of GDYPS + BSA.

In conclusion, this report provides a workable procedure for producing single genotype cultures of *C. q. fusiforme,* the causal agent of fusiform rust disease in southern pines. Past studies on fusiform rust or *C. q. fusiforme* have always had a confounding factor in that the genotype of the fungus could not be controlled within an experiment or across experiments. With the techniques presented here, the problem of fungal genotype variation can be eliminated.

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