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Effectiveness of Different *Frankia* Cell Types as Inocula for the Actinorhizal Plant *Casuarina*

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The soil bacterium *Frankia* of the *Actinomycetales*, capable of forming N_2-fixing symbiotic root nodules on a diverse array of actinorhizal plants, has several morphological forms when grown in pure culture. Fresh hydrated preparations of whole cells, hyphae, and spores were all infective on seedlings of *Casuarina* at different dilutions. Desiccated hyphae showed no infection capacity, while desiccated spores remained infective, although at a reduced level. On the basis of most-probable-number statistics, spore suspensions were 3 orders of magnitude more infective than hyphae.

There have been recent efforts to develop a viable and convenient *Frankia* inoculum for *Casuarina* root nodulation in large-scale forest plantations. While nodulation of *Casuarina* seedlings has been demonstrated with soil, crushed nodule suspensions, desiccated nodules, liquid inocula produced by various methods, and desiccated inocula, the conditions for the production of infectious inocula are still relatively unknown. One factor which may influence the infectivity of *Frankia* inoculum is the morphological characteristics of the cells.

The three typical cell morphologies of *Frankia* grown in culture, namely, vegetative hyphae, vesicles, and spore-containing sporangia, have all been described in the literature (2, 8, 11). Although it is known that the infection process in *Casuarina* spp. involves the penetration of the root hair cell wall by a growing hyphal filament (7; A. M. Berry, Ph.D. dissertation, University of Massachusetts, Amherst, 1983), it is possible that both vesicles and spores may contribute to inoculum infectivity. Several authors have demonstrated that spores taken from old cultures can germinate and form hyphae when transferred to fresh growth medium (9; R. H. Berg, personal communication). Lalonde and Calvert (5) suggested that *Frankia* spores may be responsible for the nodulation capacity of old inoculum, presumably due to their ability to remain dormant during culture senescence. Van Dijk (Ph.D. dissertation, University of Leiden, Leiden, The Netherlands, 1984) found that spore-plus nodule homogenates of *Alnus glutinosa* were about 100 to 1,000 times more infective than spore-minus nodule homogenates.

In recent studies at the Harvard Forest on inoculation of *Casuarina cunninghamiana* seedlings, our objectives have been to define the relative nodulation capacity of HFPCcI3 cell types prepared both fresh and desiccated.

Production of HFPCcI3 cell types. *Frankia* sp. strain HFPCcI3 (catalog no. HFP020203) from *C. cunninghamiana*, originally isolated and cultured by Zhang et al. (10), was used for the experiments. All cultures were incubated at 25°C and shaken at 60 rpm on a rotary shaker. Hyphal cells were produced in 125-ml Erlenmeyer flasks in 50 ml of modified BAP medium (6) containing (in millimolar concentrations) the following: sodium pyruvate, 10; potassium phosphate, 10; NH_4Cl, 5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.07; FeNa EDTA, 20; and MOPS (morpholinopropanosulfonic acid) buffer, 10; plus 1 ml of a micronutrient stock solution $liter^{-1}$ (grams $liter^{-1}$: H_3BO_3, 2.86; $MnCl_2 \cdot 4H_2O$, 1.81; $ZnSO_4 \cdot 7H_2O$, 0.22; $CuSO_4 \cdot 5H_2O$, 0.08; $Na_2MoO_4 \cdot 2H_2O$, 0.025; $CoSO_4 \cdot 7H_2O$, 0.001) and 1 ml of a vitamin stock solution $liter^{-1}$ (milligrams 100 ml^{-1}: thiamin hydrochloride, 10; pyridoxine hydrochloride, 50; nicotinic acid, 50; biotin, 22.5; folic acid, 10; calcium pantothenate, 10; riboflavin, 10). The pH was adjusted to 6.3.

Inoculum preparation. Hyphal cells were subcultured once a week for 2 months prior to harvest. Cells were washed with sterile distilled water by centrifugation at 12,000 rpm for 20 min, using a Beckman J2-21 centrifuge, and homogenized with a sterile 15-ml Wheaton glass homogenizer between each subculture and at harvest. Hyphal inoculum containing vesicles was produced as described above, except that the cells were grown in BAP medium lacking NH_4Cl containing 20 mM MOPS buffer. Spore-enriched inoculum was produced as described above, except that the cells were grown in BAP medium containing 20 mM MOPS buffer and 1 mM potassium phosphate and lacking NH_4Cl. The cultures were grown for 1 month without subculture in 500-ml Erlenmeyer flasks containing 200 ml of medium.

The spore-enriched culture described above was harvested under nonaseptic conditions. The homogenate was filtered to remove hyphae by using a Whatman no. 1 filter paper fitted into a funnel and rinsed with distilled water. The filtrate was collected, refiltered with a new filter, and then pelleted by centrifugation at 12,000 rpm for 20 min in a Beckman centrifuge; the supernatant was removed. Cytological observation of the spore preparation was made at ×40, using phase-contrast optics to check for hyphal contamination.

Seedling preparation. Seeds of *C. cunninghamiana* from seed lot 14919 (CSIRO, Canberra, Australia), collected 6 December 1988 in New South Wales, Australia, were germinated on sand covered with vermiculite and grown for 8 weeks in a growth chamber set at 16 h light and 26°C, 8 h dark and 19°C. Seeds were watered daily with deionized water until germination and then watered daily with quarter-strength Hoagland solution plus N (4). Eight weeks after germination, each seedling held in place with a cotton plug was placed in a 25-ml, 18-mm-diameter glass test tube wrapped in aluminum foil. Tubes were filled with quarter-strength Hoagland solution minus N at pH 7 and topped off

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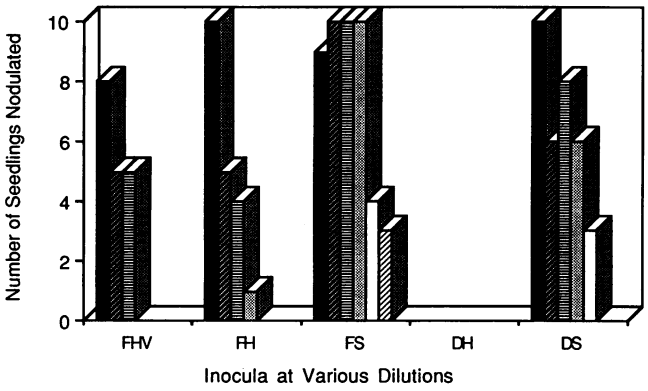

FIG. 1. Number of *C. cunninghamiana* seedlings nodulated with five types of HFPCcI3 inocula at six dilutions (n = 10). Categories: FHV, freshly prepared hyphal and vesicle inoculum; FH, freshly prepared hyphal inoculum; FS, freshly prepared spore inoculum; DH, desiccated hyphal inoculum; DS, desiccated spore inoculum. Symbols: ■, 10^{-3}; ▨, 10^{-4}; ▤, 10^{-5}; ▩, 10^{-6}; □, 10^{-7}; ▨, 10^{-8}-ml PCV per seedling.

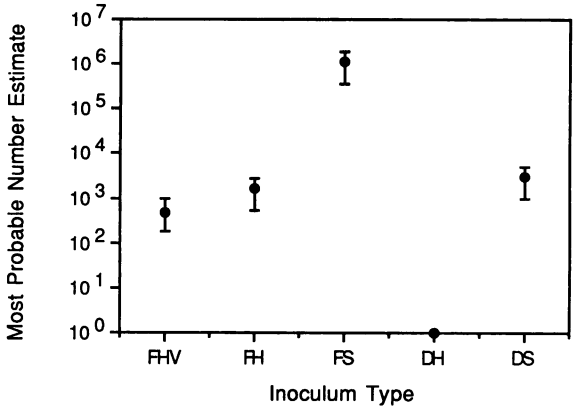

FIG. 2. Estimated most probable number of infective units in five types of HFPCcI3 inocula. Categories: FHV, freshly prepared hyphal and vesicle inoculum; FH, freshly prepared hyphal inoculum; FS, freshly prepared spore inoculum; DH, desiccated hyphal inoculum; DS, desiccated spore inoculum. Estimates were based on a 1-ml PCV quantity of inoculum following Halvorson and Ziegler (3) and Alexander (1). Data are plotted on a log scale. Vertical bars represent the estimated range at 95% confidence limits.

with deionized water once every 3 days for the duration of the experiment.

Seedling inoculation. Seedlings were inoculated with five types of HFPCcI3 inocula: (i) hyphal inoculum, (ii) hyphal inoculum with vesicles, (iii) spore inoculum, (iv) desiccated hyphal inoculum, and (v) desiccated spore inoculum. Cell populations containing hyphae alone, hyphae plus vesicles, or spores alone were transferred to a 6.5-ml graduated Kimax centrifuge tube and centrifuged at 500 rpm for 20 min in a Universal centrifuge. Inocula were prepared and diluted in 10-fold series with cell concentrations which ranged from 10^{-2}- to 10^{-7}-ml packed cell volume (PCV), each suspended in 10 ml of distilled water. Each dilution was then used to inoculate a set of 10 seedlings, which resulted in cell concentrations ranging from 10^{-3}- to 10^{-8}-ml PCV inoculum per seedling for each type of inoculum.

The desiccated inocula were identical to their freshly prepared counterparts, except that they had been desiccated by air drying for 4 days at 35°C and then rehydrated with distilled water 15 min prior to seedling inoculation. Since the various types of inocula had different production and preparation times, the initiation of cultures was staggered to reach their respective ages at the day of inoculation.

Each seedling was evaluated for nodulation in a blind fashion 7 weeks after inoculation. The most probable number of infective units in each set of inocula was then calculated by the methods of Halvorson and Ziegler (3) and Alexander (1).

Seedling nodulation. Figure 1 is a bar graph showing the numbers of seedlings nodulated, using various types of inocula prepared at six dilutions. Freshly prepared spore inoculum nodulated 100% of its respective seedlings at a dilution as high as 10^{-6}-ml PCV inoculum per seedling, and seedlings were nodulated to a limited degree even at 10^{-8}-ml PCV inoculum per seedling. Freshly prepared hyphal inoculum and desiccated spore inoculum nodulated 100% of their respective seedlings only at 10^{-3}-ml PCV inoculum per seedling (lowest dilution) and failed to nodulate seedlings at dilutions above 10^{-6}- and 10^{-7}-ml PCV per seedling, respectively. Desiccated hyphal inoculum did not nodulate seedlings at any dilution, including a set of seedlings inoculated with 10^{-2}-ml PCV inoculum per seedling (data not shown). No seedlings were nodulated in a 10-seedling, uninoculated control set.

Most probable number of infective units. The nodulation experiment made use of most-probable-number statistics developed by Halvorson and Ziegler (3) and Alexander (1). Figure 2 presents the estimated number of "infective units" in a 1-ml PCV for each type of inoculum. Vertical bars represent the estimated range at 95% confidence limits. Freshly prepared spore inoculum had almost 1,000 times more infective units than the best of the other types of inocula. Desiccated hyphae showed no infectivity, while air-dried spore preparations contained ~2×10^3 infective particles ml of PCV^{-1}.

Freshly prepared HFPCcI3 spore inoculum was demonstrably more infectious than freshly prepared hyphal inoculum. We estimated from Fig. 2 that freshly prepared spore inoculum was about 3 orders of magnitude more infectious than the other types of inocula based on most-probable-number statistics. Furthermore, spore inoculum remained infectious to a limited degree after desiccation, whereas desiccated hyphal inoculum failed to nodulate seedlings under the conditions examined. Due to the distinct morphological differences between *Frankia* hyphae and spores, however, our comparison of their respective nodulation capacities is subject to limited interpretation. The sedimentation rates of hyphae and spores are likely to be different, and thus a cell-to-cell comparison of inocula infectivity is impossible.

We estimated the number of spores in a 0.1-ml PCV of isolated spores to range from 434 to 761 million spores at 95% confidence limits, and the minimum manipulable PCV was about 0.05 ml.

Apart from stressing the importance of cytological observation and characterization of inoculum cell types prior to seedling inoculation experiments, these results suggest that, of the HFPCcI3 cell types, spores are most suitable for *C. cunninghamiana* inoculations, whether inoculum is prepared in hydrated or desiccated form. While in vitro production of sporangia and spores is a slower process relative to the production of hyphae, the conditions necessary to induce sporulation in culture and the genetic and biochemical bases

of sporulation are under study at a number of institutions. The development of more infective inoculum may be facilitated by improved methods of spore production in cultured cells.

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