The isolation of *Frankia* from nodules of *Casuarina*

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Attempting to isolate *Frankia* from *Casuarina*, we found that this endophyte exhibited two unexpected characteristics: (*i*) an inability of most infected nodule cells to produce *Frankia* colonies; and (*ii*) the absence of specific nutritional requirements in the isolation medium. These findings led us to the formulation of some principles for isolating *Frankia* from *Casuarina*.

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Au cours des essais d'isolement de *Frankia* à partir de *Casuarina*, nous avons trouvé que cet endophyte présentait deux caractéristiques inattendues: (*i*) inaptitude de la plupart des cellules infectées du nodule à produire des colonies; et (*ii*) absence d'exigences nutritionnelles particulières dans le milieu d'isolement. Ces résultats nous ont conduit à formuler quelques principes pour l'isolement de *Frankia* à partir de *Casuarina*.

Though the earliest attempts to isolate the actinomycetous endophyte from *Casuarina* nodules began at the turn of the century (see Torrey 1983), these efforts have failed up to the present time. This fact is surprising, because an increasing number of *Frankia* strains have been isolated from many genera of N_2-fixing nonlegumes (see Baker and Torrey 1979; Baker *et al.* 1981) since the first report of successful isolation of the endophyte from *Comptonia peregrina* in 1978 (Callaham *et al.* 1978).

Using the serial dilution technique, Diem *et al.* (1982) isolated five strains of *Frankia* from nodules of *Casuarina equisetifolia* collected in different parts of the world (Senegal, Guadeloupe). Although these strains exhibited the morphological characteristics of the genus *Frankia* and the ability to fix N_2 *in vitro* (Gauthier *et al.* 1981), they were unable to nodulate the host plant. We presumed that this failure could be linked to the use of an inappropriate isolation procedure; thus we decided to investigate the technique based on the use of superficially sterilized nodules directly incubated onto the nutrient medium. This technique, similar to that commonly used for isolating plant pathogenic fungi and ectomycorrhizal fungi from root tissues, had already been successfully applied to the isolation of *Frankia* from *Alnus* and *Elaeagnus* (Lalonde *et al.* 1981).

The objectives of the present study were to adapt the technique mentioned above to the isolation of the difficult endophyte from *Casuarina equisetifolia* and at the same time to determine whether the isolation of this endophyte required specific nutrients in the medium. In interpreting the results of our experiments, we attempted to formulate some concepts about the isolation of *Frankia*.

Isolation techniques

Technique No. 1: modified technique of Lalonde et al. *(1981)*

Nodule lobes were washed, superficially sterilized in a 3% aqueous solution of osmium tetroxide for 4 min (Lalonde *et al.* 1981), thoroughly rinsed in sterile water, and chopped into small pieces (ca. 0.1–1.5 mm^3) by using a sterile scalpel. Each nodule piece was transferred into a 15-mL vial containing 7 mL of semisolid (0.3% agar) Qmod medium (Lalonde and Calvert 1979). This method differed slightly from that of Lalonde *et al.* (1981) in that semisolid rather than liquid medium was used and neither phosphate-buffered saline (PBS) nor polyvinylpyrrolidone (PVP-40) was employed.

Since *Frankia* is assumed to be microaerophilic, we used the semisolid medium to maintain nodule pieces in suspension just beneath the agar surface. This technique is analogous to that devised for isolating microaerophilic *Azospirillum* (Dobereiner and Day 1976).

The vials were incubated at 25–28°C for 2 months. Vials with contaminants were discarded. In some of the remaining vials, nodule pieces carried typical *Frankia* colonies, visible under the dissecting microscope. Each colony was transferred into a 125-mL serum flask containing 15 mL of Qmod liquid medium and then broken up with a magnetic stirrer to obtain an inoculum for subculturing *Frankia*.

Technique No. 2: combination of nodule peeling and incubation on double-layer agar

To eliminate most of the contaminants harbored in the outer cell layer of the nodule lobes, we peeled away these layers as already suggested by Quispel and Burggraaf (1981). Then the nodule lobes were superficially sterilized in a 1.5% calcium hypochlorite solution for 10 min and thoroughly rinsed in sterile water. This procedure was performed along with osmium tetroxide sterilization (technique No. 1). Nodule lobes sterilized by either procedure were then aseptically cut into pieces as mentioned above. Five to 10 pieces of nodule were deposited onto a layer of 1.5% agar Qmod medium without lecithin in a petri dish. Three millilitres of the same medium, maintained at 40°C, was poured on the agar layer, thus covering the pieces of nodule. A variant of this procedure used water agar instead of the Qmod medium as the isolation medium.

Ten petri dishes thus inoculated were incubated at 25–28°C in a saturated atmosphere for preventing the desiccation of the medium during the incubation, which might last up to 2

months. Subculturing of *Frankia* colonies was performed as mentioned above.

Results

Technique No. 1

This technique was applied to the isolation of *Frankia* from nodules of a *Casuarina* hybrid (*C. junghuhniana* × *C. equisetifolia*) grown in the nursery of the Thai Forest Service in Bangkok. *Frankia* colonies appeared in 10% of the vials after 6–8 weeks of incubation. The strain designated Cj1-82 and described in detail elsewhere in this issue (Diem *et al.* 1983) was found to form effective nodules on *C. equisetifolia*.

Technique No. 2

Nodule material was obtained through the following baiting procedure. Nodules collected on the roots of adult *C. equisetifolia* trees served as inoculum for *C. equisetifolia* seedlings grown in a sterile sand–vermiculite mixture placed in a Leonard jar assembly. Nodules used for isolation were collected when seedlings were 6 months old.

Approximately 30% of the nodule pieces were contaminated. Among the remaining noncontaminated nodule pieces, ca. 70% produced *Frankia* colonies (positive nodule pieces). The percentage of positive nodule pieces remained approximately the same no matter what isolation medium was used.

Using nodules originating from Miami, FL, we isolated a strain, designated CeF1-82, on Qmod medium without lecithin. Using nodules originating from Dakar, Senegal, we isolated a strain, designated CeD1-82, on water agar.

Discussion

Sterilization of nodules

Since the outer cortical layer of actinorhizal root nodules is usually heavily contaminated with soil microorganisms the growth rate of which is always faster than that of *Frankia*, eliminating the contaminants is a prerequisite for successfully isolating *Frankia*. Different methods have been proposed to eliminate these superficial contaminants, the most convenient one being the use of osmium tetroxide (Lalonde *et al.* 1981), whereas the peeling of outer cell layers is more time-consuming (Quispel and Burggraaf 1981). Applied to *Casuarina* nodules, both methods have proven satisfactory.

Quality of the inoculum for the isolation medium

Based on our experiments in using the method of direct incubation of nodule pieces onto a solid medium, we are able to report for the first time that among the numerous cells containing the endophyte and in contact with the isolation medium, only a few (generally one or two) produced *Frankia* colonies (positive cells, Fig. 1), each colony originating from one individual cell (Fig. 2). The fact that each nodule piece bore only a very limited number of positive cells has not been explained, but one could postulate that among the peripheral cells, only a few contained *Frankia* structures capable of developing into colonies when in contact with the nutrient medium. Since we do not know the nature of these structures, we provisionally designated them (Diem *et al.* 1982) as UFF (units able to form *Frankia* colonies). The term UFF should be defined broadly to refer either to specific structures or simply to hyphae or clusters or hyphae able to grow out of the nodule. The fact that new growth of *Frankia* often originated from clusters of hyphae, also mentioned by Berry and Torrey (1979), suggests that clusters of hyphae in the nodule pieces may be more suitable than finely fragmented structures for initiating *Frankia* colonies.

The amount of UFF in the nodule may be related to the age of nodule, its physiological stage, or host plant or *Frankia* genetic determinants.

If we assume that nodules contain a limited number of UFF, the prerequisite to successful isolation would be to use a relatively large amount of inoculum made either from unprocessed nodule pieces or concentrated and purified nodule breis. Concentrated and purified nodule breis have been obtained by using Sephadex fractionation or sucrose-density fractionation (Baker and Torrey 1979) or filtration through a 20-μm screen (Benson 1982). Increase of infection units in the inoculum has been reportedly achieved by using an enrichment culture method (Quispel and Tak 1978). Following the approach of Lalonde *et al.* (1981), we used unprocessed contaminant-free nodule pieces, which generally harbour a sufficient number of UFF for successful isolation.

In this work carried out with young nodules of *C. equisetifolia*, suppressing PBS and PVP did not seem to affect the quality of the inoculum. This result should not prompt investigators to preclude the use of PVP in those cases where this procedure could protect *Frankia* structures in the nodule pieces until *Frankia* colonies are initiated.

Nutrient medium

Most media proposed for the isolation of *Frankia* are supplemented with yeast extract and other diverse compounds believed to promote *Frankia* growth, such as vitamin B12 (Baker and Torrey 1979), lecithin (Lalonde and Calvert 1979), and alcoholic extract of root lipids (Quispel and Tak 1978).

We found that successful isolation of *Frankia* from *Casuarina* nodules could be achieved by using simpler media, such as Qmod without lecithin or even water agar (technique No. 2). This result was to be expected, since in the methods used, the *Frankia* colonies remain attached to the nodule piece that provides *Frankia* with all the compounds necessary for its growth. From the absence of specific nutritional requirements observed

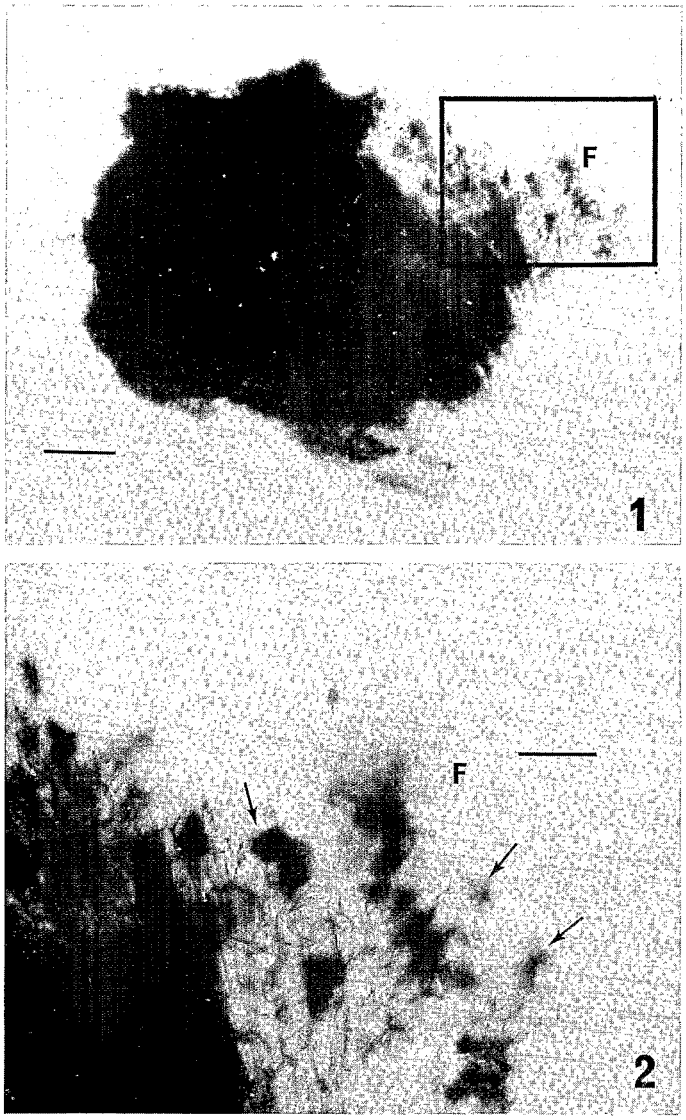

FIG. 1. Nodule piece with a colony of *Frankia* (F) growing out of a peripheral cell. Bar = 300 μm. FIG. 2. Enlargement of outlined area of Fig. 1, *Frankia* colony (F) originating from one individual cell at the periphery of the nodule piece. Note that other adjacent peripheral cells harboring the endophyte (arrows) did not form colonies of *Frankia*. Bar = 100 μm.

during the isolation phase, one must not conclude that isolated strains of *Frankia* will not exhibit such requirements when subcultured in defined media. Presently it is known that the growth of some strains of *Frankia* is stimulated by some compounds such as lipids (Quispel and Tak 1978; Quispel and Burggraaf 1981).

Our results confirmed the validity of the double-layer technique used by Quispel and Burggraaf (1981) and Diem *et al.* (1982). The main advantages of the double-layer technique over the use of liquid medium are as follows. (*i*) Contaminants do not necessarily overgrow the *Frankia* colonies. (*ii*) Microaerophilic *Frankia* colonies are not directly exposed to the atmosphere. (*iii*) *Frankia* colonies can be detected under a dissecting microscope as soon as they grow out of the nodule pieces.

Conclusion

At the present stage of our work on the isolation of *Frankia* from *Casuarina* nodules, it seems appropriate to summarize some basic concepts that should be kept in mind. First, it is well established that the growth of *Frankia* is very slow (generation time: 2–5 days), creating a high risk of overgrowth by contaminants (which always grow faster than *Frankia*) during the isolation procedure. Second, when pieces of nodules are

used as an inoculum, it does not seem necessary to prepare complex isolation media for inducing the growth of *Frankia*. Third, the nodules probably contain a fairly small number of *Frankia* units able to form colonies (UFF) on the isolation medium.

Taking into account these considerations, we suggest the following operations for isolating *Frankia* from *Casuarina* nodules: (*i*) superficial sterilization achieved by chemical (osmium tetroxide) or physical means (peeling followed by mild sterilization) to eliminate most of the contaminants without damaging the endophyte; (*ii*) preparation of a double-layer solid isolation medium with low nutrient content thus reducing the growth rate of bacterial contaminants and avoiding the overgrowth of *Frankia* by these contaminants; (*iii*) inoculation of the isolation medium with contaminant-free nodule pieces which contain a sufficient amount of UFF and provide the *Frankia* colonies originating from UFF with the specific nutrients that may be lacking in the isolation medium.

Using the techniques reported here, we isolated three strains from nodules of *Casuarina*, two of which (Cj1-82 and CeD1-82) have been already shown to nodulate *C. equisetifolia* successfully; the infectivity of the third one (CeF1-82) is currently being studied.

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