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A rapid method for the isolation and identification of *Rhizobium* from root nodules

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Summary

We developed a rapid, inexpensive method for isolating and identifying *Rhizobium leguminosarum* bv. *phaseoli* strains from bean root nodules. The method can be used for other small or medium-sized nodules such as those of clover, alfalfa and peas. With this method, one person can process 115 nodules/h. Using a simple apparatus made of plexiglass and machine bolts, we simultaneously crushed 24 surface-sterilized nodules and then transferred the crushed nodule suspension to multiple plates containing various antibiotics or a dye. This versatile and broadly applicable method complements previously described techniques, which are effective only for large root nodules such as those of soybean.

Key words: Competition; Legume; Nodules; *Rhizobium leguminosarum* bv. *phaseoli*

Introduction

Rhizobia infect the roots of legumes and induce the formation of nodules in which the rhizobia fix nitrogen. Viable bacteria are often isolated from the nodules in order to identify the infecting bacterial strain(s). Identification of the infecting strain is essential for studies of symbiotic properties such as host-range specificity and nodulation competitiveness. The feasibility of experiments that involve sampling large numbers of nodules, such as many competition studies, depends on rapid and inexpensive methods for isolating and identifying nodule occupants.

The traditional methods for isolation and identification of an infecting *Rhizobium* strain are laborious and time-consuming. Isolation typically involves handling each nodule individually while both sterilizing the nodule surface and disrupting the nodule to release bacteria. Nodule disruption is accomplished by either cutting [1], stabbing [2], or crushing [3] the nodules. The traditional methods for identification of *Rhizobi-*

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um strains based on antibiotic or phage resistance involve inoculating master plates of the nodule isolates, either of the total nodule suspension [1] or of a single colony initially isolated from the nodule suspension [4], and then plating individual isolates onto indicator plates. Recently reported methods for handling large numbers of nodules are either expensive [5] or are inappropriate for small nodules such as those formed by *R. leguminosarum* bv. *phaseoli* [6]. Large-scale sampling methods that are effective for small nodules have not been previously described.

In this paper we describe a rapid, effective and inexpensive method for isolating and identifying *R. leguminosarum* bv. *phaseoli* strains from nodules. The method depends on a simple apparatus for crushing and plating 24 nodules simultaneously. This method complements previously described techniques designed for large nodules by expanding the range of nodule sizes that can be handled in large-scale studies.

Materials and Methods

Storage and sterilization of nodules

The roots of the plants were rinsed in tap water to remove loosely adhering soil. Five to 10 nodules were removed from a plant with forceps and were placed in a microfuge tube with 1.3 ml of 20% glycerol [7]. The microfuge tube was agitated vigorously with a Vortex Mixer for 5 s and stored at −4 °C.

After 1–10 weeks, the tubes were thawed and the nodules were transferred to a tube containing 1.3 ml of 2% sodium hypochlorite. The tube was agitated with a Vortex Mixer for 1 min and the nodules were transferred to 15 ml of sterile water for 1 min. The nodules were then placed individually in the wells of a sterile, flat-bottomed, polystyrene, 96-well microtiter plate (VWR, Chicago, IL) containing 120 μl of yeast-mannitol broth (YM) [8] per well.

Multiple crusher/inoculator apparatus

The multiple crusher/inoculator apparatus (Fig. 1) was designed to be used with a 96-well microtiter plate. The apparatus consisted of an 85 × 70 × 12 mm piece of plexiglass and 24 5-cm long #632 machine bolts. The bolts were screwed into the plexiglass in 3 rows of 8 bolts and were 9 mm apart, from center to center, between the rows and 9 mm apart within the rows. After assembly, the bolts were rinsed once with acetone to remove the manufacturer's coating.

Isolation and identification of nodule bacteria

The multiple crusher/inoculator apparatus was dipped in ethanol, flame-sterilized, positioned in the wells of the microtiter plate, and gently rocked to crush the nodules. Nodules that were missed by the apparatus were crushed individually with a flame-sterilized aluminum rod. The multiple crusher/inoculator apparatus was then dipped into the wells and the 24 drops of bacterial suspensions carried on the bolts were dropped onto a YM agar plate (Fig. 2). Repeated transfers were then made to plates containing indicator antibiotics or the dye Calcofluor [9]. In this manner, the isolates in half of a microtiter plate (48 wells) were placed on each agar plate (Fig. 2).

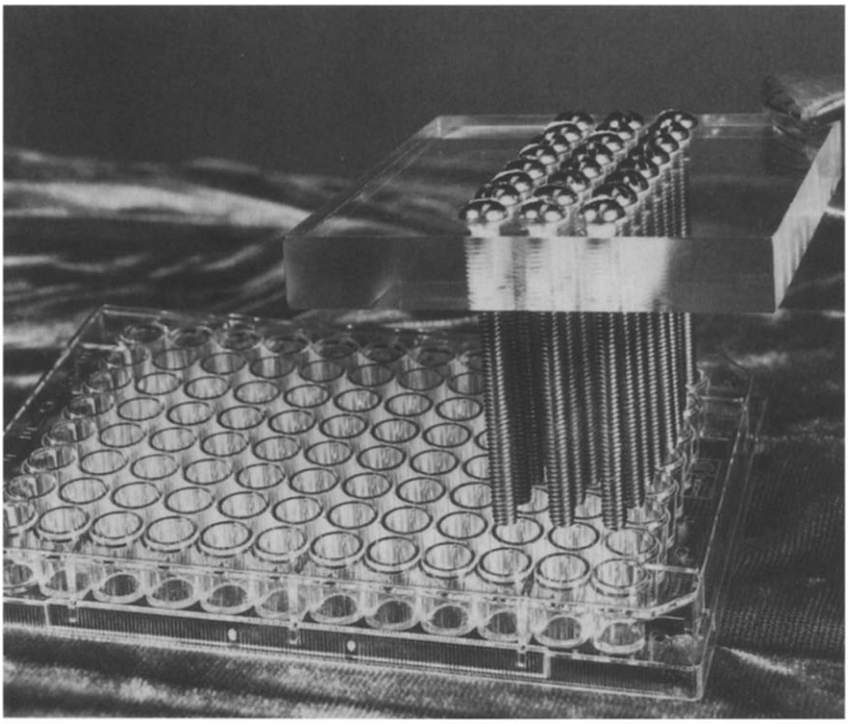

Fig. 1. The multiple crusher/inoculator apparatus in a microtiter plate.

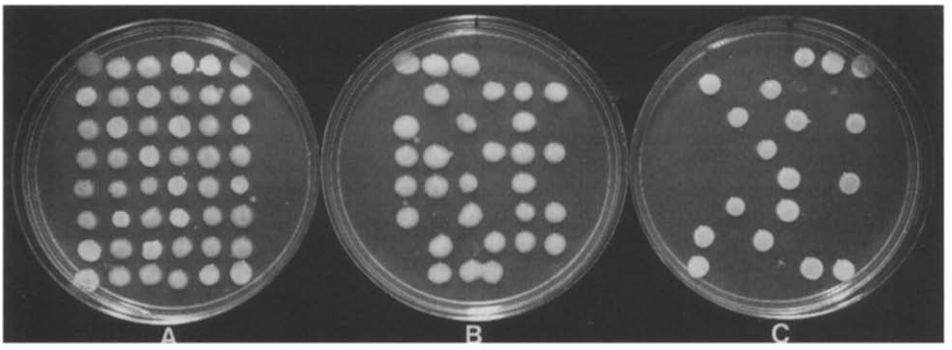

Fig. 2. Isolates from 48 nodules on a master plate and 2 different indicator plates after 2 d at 30 °C. (A) YM medium; (B) YM medium supplemented with 250 μg/ml streptomycin; and (C) YM medium supplemented with 200 μg/ml spectinomycin. Note that all the isolates grew on the master plate and on 1 antibiotic; none of the isolates grew on both antibiotics.

Results and Discussion

The proportion of nodules containing viable bacteria after storing and processing the nodules depended on the storage procedure. Viable bacteria were isolated from

90% of the nodules when the nodules were completely immersed in 20% glycerol, frozen, and then thawed before processing. When the nodules were incompletely immersed, viable bacteria were isolated from fewer nodules. Freezing the plants and then thawing, removing and processing the nodules resulted in recovery of viable bacteria from only 48% of the nodules. Storing the nodules at room temperature with a drying agent and then soaking them in 1% NaCl for 2–3 h [10, 11] before processing resulted in viable bacteria from only 65% of the nodules.

Vigorous agitation of the nodules in a dilute sodium hypochlorite solution eliminated the need for a wetting agent and effectively sterilized nodules with highly convoluted surfaces such as those formed by *R. leguminosarum* bv. *phaseoli*. Surface contamination was checked by sterilizing 96 nodules in hypochlorite as described, gently agitating each nodule in 120 μl of YM in a well of a microtiter plate, and placing a 3 μl drop of the YM solution onto a YM plate. Among the 96 drops, 1 colony grew in each of 6 drops and confluent colonies grew in 1 drop after 2 d of incubation at 30 °C. No growth was observed in the other 89 drops. We only scored as positive those drops from crushed nodules that had more than 10 colonies or confluent growth, since 10 or fewer colonies could be accounted for by spontaneous mutation to antibiotic resistance. Thus, surface contamination from nodules sterilized by this method was about 1%.

The multiple crusher/inoculator apparatus described here disrupts the nodules sufficiently to result in confluent growth when a drop of the suspension is placed on a YM agar plate. A major advantage of inoculating with this apparatus over replica plating bacterial colonies is that the apparatus delivers virtually identical samples to each indicator plate. The drops that are delivered to each of the replicate plates all have the same volume (3 μl) and contain the same concentration of bacteria. All of the drops contain between 10^4 and 10^7 bacteria. The rigidity of the apparatus permits delivery of the drops in a defined pattern and thus eliminates the time required in other methods for individually localizing samples on a grid. Furthermore, the apparatus places 48 samples on each plate and thus reduces the number of petri plates required for an experiment compared to other nodule sampling methods which typically place only 10–16 samples on a plate [6].

The time that is required for isolating and plating the isolates from 96 nodules onto 3 indicator plates is 50 min: 3 min for filling the microtiter plate with YM medium; 35 min for sterilizing the nodules and loading them into the microtiter plate; 8 min for crushing; and 4 min for transferring the bacterial suspensions to the 3 indicator plates. This is equivalent to 115 nodules/h of labor. In contrast, when each nodule was crushed individually and streaked for isolated colonies, a maximum of 12 nodules were processed in 1 h. The major time-saving steps presented here are the handling of many nodules at once during sterilization, crushing and plating, and the identification based on the properties of the total population of bacteria in the crushed nodule suspension rather than on those of a single representative colony. This method enabled us to increase our sampling size from 500 nodules in each of 2 field seasons to 8000 nodules in a third field season.

In summary, we have described a rapid and inexpensive method for isolating and identifying infecting *Rhizobium* strains from nodules of a wide range of host legumes. The method can be used for any small or medium-sized nodules such as clover, alfalfa or pea nodules, all which have diameters in the range of 1–8 mm, in contrast to previ-

ously described methods that are useful only for larger nodules, such as those formed on soybeans. The method described here can also be easily modified for other identification techniques such as identification by sensitivity to phage, hybridization to labelled DNA probes, or binding of fluorescent antibodies.

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