Correct citation: Graham, P.H. & Harris, S.C. (Eds.) 1982. Biological Nitrogen Fixation Technology for Tropical Agriculture: Papers Presented at a Workshop Held at CIAT, March 9-13, 1981. Cali, Colombia, Centro Internacional de Agricultura Tropical, 768p.

APPLICATION OF INHERENT ANTIBIOTIC RESISTANCE TO ECOLOGICAL STUDIES OF RHIZOBIA

O.P. Rupela,[1] D.P. Josey,[2] B. Toomsan,[1] S. Mittal,[1] P.J. Dart,[1] and J.A. Thompson[1]

Summary

The inherent antibiotic resistance technique shows promise for use in ecological studies. The technique involves the use of readily available chemicals for growing rhizobia, antibiotics and a simple, easily manufactured multi-inoculator. The main requirement of the method is precise maintenance of conditions throughout the experiment for all variables and laboratory processes. These precautions are discussed.

In a study of 473 field isolates from a trial inoculated with a streptomycin (str 200) marked mutant, the use of 30 characteristics (10 antibiotics x 2-4 concentrations) classified the strains into 203 groups when all characteristics had to match perfectly, and into 119 groups when one mismatching characteristic was allowed. The 22 isolates having str 200 resistance were placed in three groups with no mismatches, and into two groups with one mismatching character allowed.

INTRODUCTION

The success of introduced *Rhizobium* strains in field trials has been monitored using both serology and strains marked with antibiotic resistance (Read, 1953; Dudman & Brockwell, 1968; Schwinghamer & Dudman, 1973). Neither of these techniques, however, gives much information concerning the composition of the indigenous population of *Rhizobium*. Using the inherent antibiotic resistance technique described by Josey *et al.* (1979), the indigenous soil population of *Rhizobium* has been shown to be heterogeneous (Beynon & Josey, 1980). This technique could be used in examining some current

[1] CP No. 43, ICRISAT, Patancheru P.O., Andra Pradesh 502-324, India.
[2] John Innes Institute, Colney Lane. Norwich NR4 74H, England.

problems in field inoculation trials: the heterogeneity of indigenous populations, changes in the population over cropping season and with particular agricultural practices, and, in some cases, the reason for the success of particular inoculant strains. This paper discusses precautions to be observed in adapting the technique to local conditions, and reports on application of the technique to study rhizobia nodulating chickpea (*Cicer arietinum* L.) at ICRISAT.

SOURCES OF ERROR IN THE INHERENT ANTIBIOTIC RESISTANCE METHOD

Every effort must be made to maintain rigid control of the experimental conditions. If the test conditions are not completely standardized, it will be very difficult to draw any conclusions about the relationship between strains examined in different test series. Potential sources of error using this technique are:

Medium composition: The concentration of all constituents of the growth medium must be constant, and media should always be prepared using the same grade and brand of reagents. The number of ions available in one make of yeast extract may be widely different from those in another brand, and this can affect strain growth and antibiotic resistance differentially.

Medium sterilization and melting: Growth medium should always be sterilized and melted in the same way. If the medium is heated for different periods of time or at different temperatures, its composition may also vary, and hence affect growth.

Antibiotics: The same supplier should be used, as the strength and formulation of antibiotics can vary between manufacturers. The potency of nearly all antibiotics will decrease with age, particularly when made up into stock solution. so large amounts of stock solution should not be prepared. Repeated freezing and thawing of antibiotics should also be avoided as much as possible.

Mixing of antibiotics in media: When mixing the antibiotic with agar medium the temperature should be kept at 60° in a water bath. Once the plates are poured some antibiotics also will start to lose activity; thus, plates should be inoculated as soon as the whole set of plates is ready. If the agar is cool during mixing (c. 45°C), an even distribution of antibiotic throughout the medium may not be achieved, and strict comparisons between plates will not be possible. It is very important that the antibiotic concentrations in the medium are accurately reproduced on each occasion.

Inoculum condition: Sensitivity to some antibiotics (such as penicillin) may depend on cell growth phase, and it is very desirable to use inocula of a reasonably uniform stage of growth. Cells in the stationary phase may survive exposure to an antibiotic to which they are normally considered sensitive and then commence to grow again after concentration of antibiotic in the medium has decreased below the threshold value for activity against that particular strain.

Thickness of plate: If the thickness of medium in the plates varies, then the colony morphology will be influenced; e.g., slime may only be produced by large colonies making it difficult to assess the difference between control and antibiotic plates. if they are of different thickness. Plates with bubbles should also be discarded because of lack of homogeneity.

Drying of plates: As plates should be used immediately, drying may not be practical unless a laminar flow sterile hood is available. If plates are dried the same procedure must be used on every occasion.

Contamination: Contaminated plates should be discarded, as waste products from the contaminants may be synergistic with, or destroy, the antibiotics, and these effects may permeate the whole plate; not just the region where the contaminant is growing. For similar reasons, fast- and slow-growing strains of *Rhizobium* should not be tested on the same plate.

AN APPLICATION OF THE TECHNIQUE TO RHIZOBIA NODULATING CHICKPEA AT ICRISAT

In an experiment with chickpea at ICRISAT, in which the mutant strain 9036, resistant to 200μg/ml streptomycin was used, 473 nodule isolates were obtained that lacked this resistance, and 22 were found with resistance to streptomycin. Inherent antibiotic resistance studies were undertaken to characterize these strains.

The technique used was as follows: Yeast extract mannitol agar (YMA), was made up accurately, sterilized, and kept at 60-65°C ready for use. Just before pouring the plates, the required volume of antibiotic stock solution (see Table 1) was added to the known volume of medium in the flask and swirl-mixed to ensure its proper distribution in the medium.

The same volume of medium was added to each petri dish, using either a sterile measuring cylinder or a sterile graduated beaker; for 9 cm diameter glass petri dishes, 30 ml was used. The plates were then poured on a flat surface to achieve uniform thickness.

Rhizobium strains used for strain typing were grown on YMA slopes in McCartney bottles, and as soon as growth was seen (usually four to five days with *Cicer* rhizobia) the cells were suspended in sterile, 20% (v/v) glycerol, dispensed in small quantities suitable for one day's testing, and stored in a deep freeze at -10°C.

The antibiotic test plates and controls were inoculated with a pronged, multiple inoculator (Josey *et al.*, 1979). Glycerol-stored cultures were diluted with sterile distilled water to give approximately 10^5 cells/ml and placed in the wells of the pin inoculator. At this concentration each prong of the inoculator transferred aproximately 10^3 rhizobia to the test plates. After incubation at about 28°C for 7 days the growth on antibiotic medium was compared with

TABLE 1: List of antibiotics and their concentrations.[1]

Antibiotics [2]	Concentrations (mg/1)
Carbenicillin	1.0, 2.5, 5.0
Erythromycin	1.25, 2.5, 10
Kanamycin	2.5, 10, 20
Nalidixic acid	2.5, 10, 15
Neomycin	2.5, 10, 15
Polymyxin	5, 10, 20
Rifampicin	0.25, 0.5, 2.5
Streptomycin	2.5, 10, 20, 200
Tetracycline	0.1, 0.5
Vancomycin	1.25, 2.5, 10

[1]All antibiotics are from Sigma except carbenicillin which is from 'Pyopen', Beecham.
[2]Antibiotic solutions were made in sterile deionized water, except erythromycin (in ethanol) and nalidixic acid (in 1 M NaOH).

that on control plates. Colony growth was scored numerically (1: no growth; 2: weak growth; 3: good growth) so that the results were amenable to computer analysis.

When the 473 isolates were scored on the basis of resistance to 10 antibiotics at 2-4 concentrations. 203 distinct groups were found. When one mismatch in the array of tests was permitted, 119 groups were distinguished. Samples of strain 9036 (reference culture) could be separated into two groups by inherent antibiotic resistance, while streptomycin-resistant field isolates fell into these two groups, plus one other.

When one mismatch was allowed, all reference cultures fell intc one group as did 21 of the 22 streptomycin resistant field isolates.

The results demonstrated again the heterogeneity of soil populations. Characterization of the properties of the different groups of isolates, and of heir frequency in soil, could help explain the basis for their competition with

Josey, D.P., Beynon, J.L., Johnston, A.W.B., & Beringer, J.E. (1979) *J. Appl. Bacteriol.* 46. 343-350.

Read, M.P. (1953) *J. Gen. Microbiol.* 9, 1-3.

Schwinghamer. E.A. & Dudman, W.F. (1973) *J. Appl. Bacteriol.* 36, 263-272.