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PRELIMINARY ECOLOGICAL STUDY OF *Rhizobium*: SELECTION OF ANTIBIOTIC RESISTANT MUTANTS OF *Rhizobium* PMA295 NODULATING *Sesbania sesban*

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ABSTRACT

This study was designed to provide strains for studying the ecology of *Rhizobium* when it was used to inoculate seed sown in the soil or in the field. Naturally occurring antibiotic resistant mutant strains of *Rhizobium* PMA295 nodulating *Sesbania sesban* were selected by antibiotic selection method. The isolates should be as effective in nitrogen fixation as the parental wild-type strain. Authentication of mutant isolates showed that these rifampicin mutant isolates could nodulate test plant and most nodules formed by the rifampicin 50 ppm and streptomycin 500 ppm mutant isolates were red in colour. These twenty isolates can be regarded as rhizobia. The most effective mutant, isolate PMA295 selected on rifampicin 50 ppm was also resistant to rifampicin 60, 70, 80, and 90 ppm. The effectiveness of most mutant strains was not significantly different from their parent strain. The rifampicin 50 ppm mutant strain of Rhizobium PMA295 could be used as inoculant for ecological study.

[*Keywords: Rhizobium*; strains; ecological characteristics; resistant mutant; rifampicin; streptomycin; *Sesbania sesban*]

INTRODUCTION

Studies of the *Rhizobium* ecology are depending on the techniques to identify strains occupying nodules and surviving in the soil. Until the recent development of DNA probes and PCR techniques (Young *et al.*, 1991), the numeration of specific *Rhizobium* strains in nonsterile soil and in the rhizosphere relied on serological or spontaneous antibiotic resistant mutant methods. The property of antibiotic resistance is a distinguishing characteristic that permits identification of the strain. These mutants can be detected by exposing strains to antibiotic containing media.

Many rhizobia are naturally resistant to low levels of various antibiotics (Graham, 1963). The rate of mutation for *Rhizobium* ranges from 1 in 10^5 to 1 in 10^7 (Somasegaran and Hoben, 1994). Antibiotic resistant mutants are valuable for both laboratory and field experiments, particularly the latter because the antibiotic aids in isolation and enrichment of the resistant strains, i.e., most bacterial contaminants can be eliminated during isolation of rhizobia (Schwinghammer and Dudman, 1980). This study aims to select and to evaluate antibiotic resistance of rhizobial strain PMA295 nodulating *Sesbania sesban* for nitrogen fixation potential.

MATERIALS AND METHODS

Development and authentication of antibiotic resistant mutants. Rhizobial strain PMA295 effectively nodulating *S. sesban* was used in the experiment. Two antibiotics, rifampicin and streptomycin were used to select mutants. Parent, wild-type *Rhizobium* was grown in Yeast Mannitol Broth (YMB) to population of 10^8 cell ml^{-1}. An aliquot of 0.1 ml of this culture was then spread-plate on Yeast Mannitol Agar (YMA) containing 50 ppm rifampicin. The rifampicin was firstly dissolved in methanol and 500 ppm streptomycin was dissolved in sterile distilled water. Control plates were parent strains grown on YMA without antibiotic. The plates were incubated within 3-7 days at 24°C.

To test the stability of mutant isolates, ten colonies picked from the antibiotic containing plates were successively subcultured for nine times on YMA without antibiotics. Those isolates were then retested for antibiotic resistance by streaking them on YMA media containing antibiotics. The ability of *Rhizobium* to nodulate *S. sesban* was used to authenticate them as resistant mutant isolates. The tests were done in sterile sand culture in the greenhouse as follow: Seeds of *S. sesban* were scarified and surface-sterilized with concentrated sulphuric acid for 15 minutes, followed by five times rinsing with sterile distilled water. The seeds were then germinated on 1% sterile water agar prior to transplanting into the sand culture. Four plants were grown in 12.5 cm diameter pots and then thinned to two plants. The plants were grown for 5 weeks. Treatments were inoculated and uninoculated plants with antibiotic resistant mutant isolates. Each treatment had two replications. At harvest, plant colour, shoot dry

mass, nodule location, nodule number, nodule colour, and nodule dry mass were recorded. The mutant strains of PMA295 selected on rifampicin 50 ppm were streaked on rifampicin containing medium at concentrations of 60, 70, 80, and 90 ppm in the same YMA media. It was considered necessary since naturally occurring rhizobia were frequently resistant to rifampicin 50 ppm. The isolates confirmed as *Rhizobium* were tested for effectiveness on the same host in sand culture.

Effectiveness of antibiotic resistant mutants. *S. sesban* seeds were scarified and surface-sterilized as described before. Ten seeds were planted in the sterilized sand in 15 cm diameter pots then thinned to four plants per pot. Before autoclaving, nutrient solution containing P 0.161, Mg 0.53, K 1.58, Ca 1.35 mM and Fe 8.9, Cu 0.79, Zn 1.1, Mn 1.4, B 9.2, and Mo 0.1μM were added to moisten the sand. The nutrients were added to the pots whenever they were watered by drip feeder on automatic timer. The plants were inoculated by antibiotic resistant mutant strains of *Rhizobium* by pipetting 2 ml of broth culture with population of 10^8 cell ml^{-1} at planting. The plants were grown for 8 weeks in a glass-house with evaporative cooling and heating to minimize temperature fluctuation. One selected parent strain, 10 selected mutant strains for each antibiotic, and uninoculated control without and with 6 mM KNO_3 were used for the studies with four replication and randomized within each replicate block. Shoot dry mass, nodule number, and nodule dry mass were measured at 8 weeks.

RESULTS

Authentication of antibiotic resistant mutant isolates as Rhizobium. Isolates grown on streptomycin were larger in diameter than those grown on rifampicin containing media or on media without antibiotics (wild-type colonies). It was shown that after nine subculturing, the mutants on YMA media without antibiotics, grew abundantly with the same phenotypes with the mutants grown on the antibiotic media as the original mutant isolates. Table 1 shows that all isolates tested formed nodules on *S. sesban*. One replicate of streptomycin resistant (isolate no.1) failed to form nodules. Most of the nodules formed by the rifampicin mutant isolates were red in colour. Nodules on one replicate streptomycin resistant (isolates no. 1, 4, 5, and 6) and both replicate of

Table 1. Authentication of mutant isolates on *Sesbania sesban*.

Inoculum	Nodulation			Plant colour
	+/-	Colour	Number per pot	
Rif-50 resistant isolates				
1	++	RR	14,17	G, G
2	++	RR	9,14	Lg, Lg
3	++	RRw	11,24	Lg, Lg
4	++	RR	8,18	G, G
5	++	RR	13,7	G, Lg
6	++	RR	10,19	G, G
7	++	RR	15,8	Lg, Lg
8	++	RR	13,7	Lg, Lg
9	++	RR	13,11	Lg, G
10	++	RR	8,16	Y, Lg
Str-50 resistant isolates				
1	+-	RRw	14,17	G, G
2	++	RR	12,17	Lg, Lg
3	++	RR	18,15	Lg, Lg
4	++	RwR	13,19	G, G
5	++	RwR	15,13	G, Lg
6	++	RRw	27,14	G, G
7	++	RR	19,10	Lg, Lg
8	++	RwRw	25,28	Lg, Lg
9	++	RR	11,20	Lg, G
10	++	RR	12,11	Y, Lg
Parent	++	RR	19,17	G, G

++ = two replicates nodulated; + = one replicate nodulated; RR = red colour (two replicates); RRw = red and white colour in one plant; GG = green (two replicates); LgG = light green (one replicate); Y = yellow.

streptomycin resistant isolate no. 8 were white. These ten isolates can therefore be regarded as rhizobia. The most effective mutant isolate of PMA295 selected on rifampicin 50 ppm (isolate no. 3) was also resistant to rifampicin 60, 70, 80, and 90 ppm.

There were significant differences in shoot dry mass among the isolates tested. Largest shoot dry mass was obtained by isolate no. 1 resistant to streptomycin 500 ppm (0.08 g pot $^{-1}$) and this was not significantly different to isolates no. 2 and 3, and rifampicin mutant isolates no. 1, 2, 3, 4, 6, and 7. These isolates did not differ from the parent strain (P) which was 0.09 g pot $^{-1}$ (Fig. 1). Nodule number was not correlated with shoot dry mass for isolates resistant to streptomycin (r = 0.10) or rifampicin (r = 0.71).

Effectiveness of the antibiotic resistant mutant strains. Ten isolates of rifampicin resistant mutant and all isolates of streptomycin resistant mutants except isolates no. 9 and 10 produced shoot dry mass that was not significantly different from their parent strain. Plants supplied with combined nitrogen produced significantly larger shoot dry mass (1.743 g pot^{-1}) than parent and mutant strains which were in a range of 0.119-0.967 g pot^{-1} (Fig. 2). There was not significantly difference in nodule dry mass for rifampicin and streptomycin mutant isolates except for streptomycin resistant isolates no. 9 and 10 (Fig. 2). This was reflected in plant colour, where these two isolates produced yellow to light green coloured plants (Table 1).

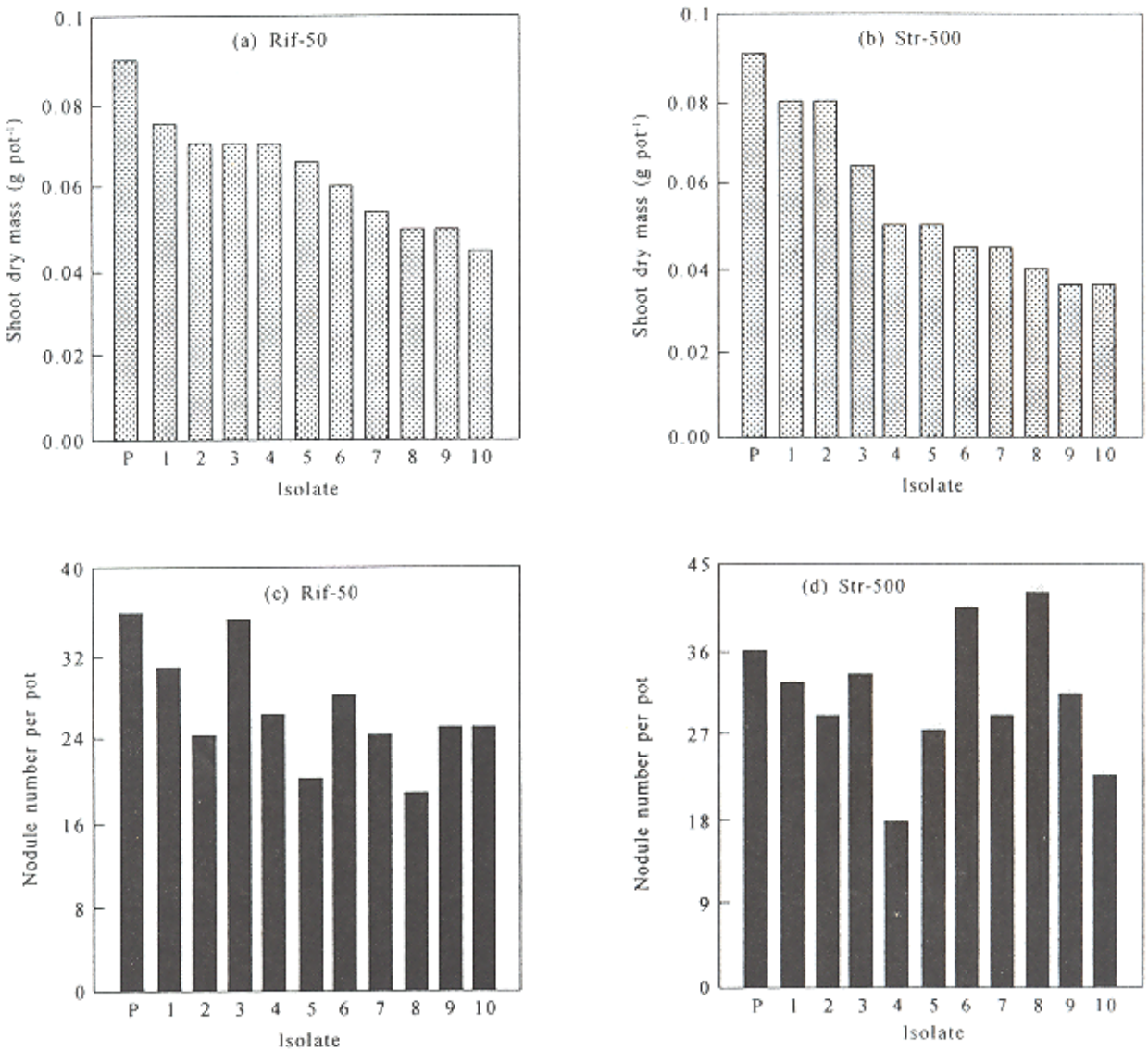

Fig. 1. Authentication of 10 rifampicin and 10 streptomycin resistant mutant isolates of *Rhizobium* PMA295 on *Sesbania sesban*; P = the wild-parent strain of *Rhizobium* PMA295, 1-10 = rifampicin or streptomycin resistant mutant isolates.

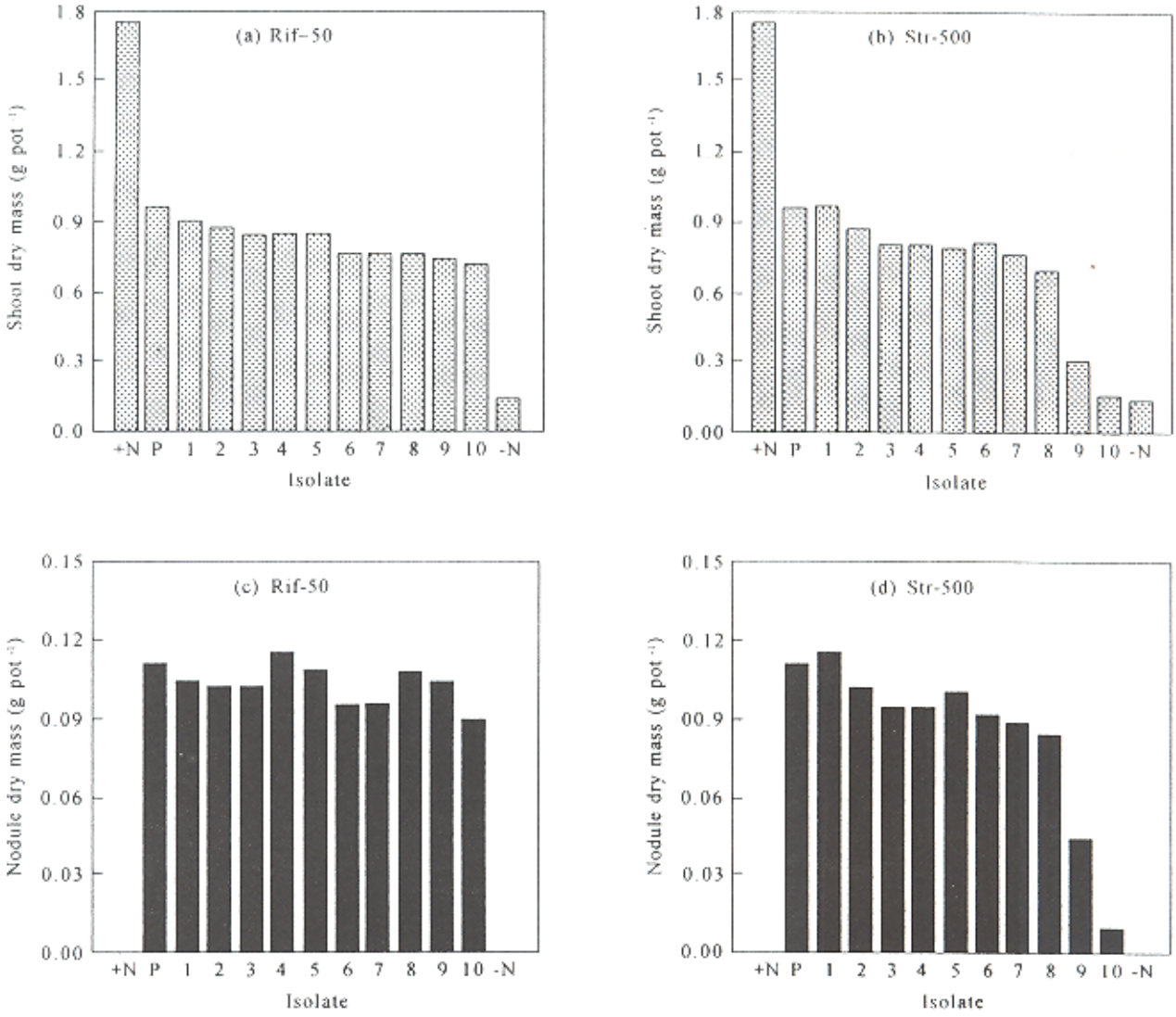

Fig. 2. Effectiveness of 10 rifampicin and 10 streptomycin resistant mutant isolates in comparison with the wild-parent strain of PMA295 on *Sesbania sesban*; +N = plants received 6mM NH_4NO_3 in the nutrient solution daily, P = the wild-parent of PMA295, 1-10 = rifampicin or streptomycin resistant mutant isolates, -N = control.

DISCUSSION

The antibiotic resistant mutants were intended to study the ecological characteristics of the parent strain. Therefore, it was important that the mutants selected should resemble the parent strain in many characteristics as possible. No differences in growth characteristics were observed between the parent strain and any rifampicin resistant strains in this study. However, there were two ineffective streptomycin resistant strains, therefore these mutant strains could not be used for studying the ecology of parent strain. Schwinghamer and Dudman (1980) reported similar differences for mutants resistant to spectinomycin, which nodulated subterranean clover. Antibiotics such as streptomycin or spectinomycin inhibit RNA protein synthesis and rifampicin inhibits protein polymerase. In *Rhizobium*, mutants resistant to these antibiotics were less likely to have defect in symbiosis (Schwinghamer. 1968). Pankhurts (1977) found that there was no relation between loss of effectiveness and resistance to particular groups of antibiotics including rifampicin and streptomycin. Rifampicin 50 ppm mutant strain *of Rhizobium* PMA295 was also tested on 60, 70, 80, and 90 ppm of rifampicin to anticipate if native strains would have

the same resistance on antibiotics used (rifampicin 50 ppm). Purwantari (1994) reported that native rhizobial strains resistant to rifampicin 50 ppm in some soil were unable to grow in media containing higher rifampicin concentration. Therefore in this condition, rifampicin 50 ppm mutant strain of *Rhizobium* PMA295 can be used as inoculant in soil media or in fields to study their fate in the soil since native rhizobial strains presence in the soil would not be resistant to antibiotics and need to be tested. The implementation of this study would be to assess the need for inoculation or reinoculation seed or soil with rhizobial strains.

CONCLUSION

Rhizobium PMA295 produced resistant mutant strain on both antibiotics, rifampicin 50 ppm and streptomycin 500 ppm and their effectiveness did not differ with parent strain. Strain PMA295 rifampicin 50 ppm was also resistant to 60, 70, 80, and 90 ppm of rifampicin.

REFERENCES

Graham, P.H. 1963. Antibiotic sensitivities of the root nodule bacteria. Aust. J. Biol. Sci. 16: 557-559.

Pankhurst, C.E. 1977. Symbiotic effectiveness of antibiotic-resitant mutants of fast and slow-growing strains of *Rhizobium* nodulating *Lotus* species. Can. J. Microbiol. 23: 1026-1033.

Purwantari, N.D. 1994. The biology and nitrogen fixation of some shrub legumes. PhD Thesis. Univ. of Queensland, Australia.

Schwinghamer, E.A. 1968. Loss of effectiveness and infectivity in mutants of *Rhizobium* resistant to metabolic inhibitors. Can. J. Microbiol. 14: 355-367.

Schwinghamer, E.A. and W.F. Dudman. 1980. Methods for identifying strains of diazotrophs. p. 337-365. *In* F.J. Bergensen (Ed.) Methods for Evaluating Biological Nitrogen Fixation. John Wiley & Sons Ltd., New York.

Somasegaran, P and H.J. Hoben. 1994. Handbook for Rhizobia: Methods in legume-rhizobium technology. Springer-Verlag. New York. 450 pp.

Young, J.P.W., H.L. Downes, and B.D. Eardly. 1991. Phylogeny of the phototropic *Rhizobium* strain BT Ail by polymerase chain reaction based sequencing of a 16S rRNA gene segment. J. Bacteriol. 174: 2271-2277.