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Effect of Acridine and Ascorbic Acid on Rhizobia of Legume Trees

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Abstract: The present study aimed to induce a great variation exists among *Rhizobium* spp. that nodulate *Leucaena leucocephala* and *Sesbania sesban*, through the treatment with acridine and ascorbic acid and selection of strains for efficiency of the symbiotic association appears possible. Bacteria were isolated from nodules of both hosts growing in Egyptian soil, subcultured and verified to be rhizobia. The isolates varied significantly in their resistance to antibiotics and NaCl, their growth on different carbohydrate sources and their effect on the pH of culture media. Most isolates showed intermediate antibiotic resistance, the capacity to use numerous carbohydrates and a neutral to acid pH response. The mean generation times of these isolates ranged from 4.813 to 6.437 and 4.600 to 6.523 for *Sesbania sesban* and *Leucaena leucocephala*, respectively. Both acridine orange and ascorbic acid appeared genotoxic hazards effect on all rhizobial strains examined here. They demonstrated a dose-response for decreasing cell survival at the levels that are not excessively toxic to the bacteria. The standard assay with pie-incubation was quite toxic to the bacteria than plate incorporation test. Acridine orange and ascorbic acid shows an increase in the number of auxotrophic mutants over the spontaneous value this is evidence for their mutagenicity caused by DNA damage. The biochemical mutants obtained in this study were identified using nine plates of minimal medium each was supplemented with different combinations of four growth factors. From the results reported here, it can be concluded that acridine mutagenesis is due to an enhancement of mismatch repair. In addition, ascorbic acid may be mutagenic and cytotoxic through the generation of hydrogen peroxide.

Key words: Acridine, antibiotic resistance, ascorbic acid, auxotrophic mutant, generation time, mutation frequency, mutation rate, *Rhizobium*, salt stress

Introduction

Successful nodulation and nitrogen fixation by legumes inoculated with *Rhizobium* strains depend on genetic and environmental factors. Once introduced, *Rhizobium* bacteria must multiply in the rhizosphere and infect their host plant to initiate the symbiotic process. Success depends on competitiveness, ability to survive in the soil and incompatibility with the host (Dowling and Broughton, 1986). Many effective strains have been reported to be poor competitors and may, consequently, form very few nodules in the presence of ineffective strains (Trinick, 1982). Sensitivity to antibiotics varies between species and it has been suggested that such variation may be a useful taxonomic character (Graham, 1963). A potential problem with the use of antibiotic-resistant mutant strains for ecological studies is that they may vary in other important characteristics, including competitiveness for nodule formation and ability to fix nitrogen. Therefore, it is very important to compare resistant strains with wild-type strains if the genetically marked strains are to be used in experiments designed to draw inferences concerning the ecology of the parental strains.

Recently, Thomas and MacPhee (1987) have reported that acridine-induced frame shift mutagenesis might be subject to glucose repression. Glucose indirectly controls the expression of a number of catabolic operons (e.g., *Lac*, *gal*, *ara*) by lowering the intracellular concentration of cyclic AMP (cAMP). In a previous study, Pons and Muller (1989) had shown that upon treatment with 9-amino acridine (9AA) in non-growth medium of log-phase cells of *E. coli* K12/343/113, there was an increase in *nad*-113→ Ned^+ reversion of more than 5 orders of magnitude. Using this sensitive frame shift marker, Pons and Muller (1989) showed that the glucose effect in acridine-induced mutagenesis is actually an "energy effect" and that it is due to mismatch repair. *In vitro*, mutagenesis bioassays are used to identify environmental chemicals that are potential genotoxic hazards for man. Recent reports have indicated that ascorbic acid, at high concentrations, may be mutagenic (Galloway and Painter, 1979) and cytotoxic in these *in vitro* test systems. L-Ascorbic acid (vitamin C) has been found to be capable of modifying the properties of DNA (McCarty, 1945). Ascorbate can inactivate several phages by induction of single-strand breaks in their DNA (Murata *et al*., 1986) and can induce multiple breaks in the chromosomes of repair-deficient mutants of *E. coli* (Van Sluys *et al*., 1986). In this study, the genetic diversity of legume trees rhizobia concerning sensitivity to antibiotics and tolerance to salt, as well as, the mutagenic effect of acridine and ascorbic acid in different *Rhizobium* strains were determined.

Materials and Methods

Bacterial strains and cultures: *Rhizobium* strains which isolated and used in the present study are listed in Table 1. Tryptone-yeast extract medium (TY) was used as a complete medium to ensure the independence of mutations according to Beringer (1974). Liquid growth medium (VS) was used to allow expression of mutations according to Pain (1979). Minimal medium (MM) was used according to Balassa (1963) and Murphy and Elkan (1963) for mutants isolation and identification through supplemented with a suitable concentration of the appropriate requirements. Mineral medium was also used for carbon source utilization test according to Milnitsky *et al*. (1997) by replaced mannitol with the appropriate carbon source at a final concentration of 1% (w/v). Yeast extract mannitol medium (YEM) was used for culture maintenance according to Allen (1959). YEM with Congo-red contained of 10 ml of Congo-red (0.25 g of Congo-red in 100 ml DW) were added to each

Table 1: *Rhizobium* strains from different locations in Egypt and used in this study

Legume host (source of rhizobia)	Location	Designation
Sesbania sesban	Far. Fac. of Science, Mansoura Univ.	$FFSMU_1$
Sesbania sesban	Far. Fac. of Agric., Mansoura Univ.	$FAFMU_2$
Sesbania sesban	Far. Fac. of Agric., Mansoura Univ.	$FAFMU_3$
Sesbania sesban	Far. Fac. of Agric., Mansoura Univ.	$FAFMU_4$
Sesbania sesban	Far. Fac. of Agric., Mansoura Univ.	$FAFMU_5$
Sesbanie sesban	Far. Fac. of Agric., Mansoura	$FAFMU_6$
Sesbania sesban	Fac. of pharmacy., Mansoura Univ.	$FPMU_7$
Sesbania sesban	Fac. of Art., Minia Univ.	$FFAMU_8$
Sesbania sesban	Agric., Res. Center, Giza	$ARCG_9$
Sesbania sesban	Agric., Res. Center, Giza	$ARCG_{10}$
Sesbanla sesban	Agric., Res. Center, Giza	$ARCG_{11}$
Sesbanie sesban	Agric., Res. Center, Giza	$ARCG_{12}$
Sesbania sesban	Sakha Agric. Res. Station	$SARS_{13}$
Sesbania sesban	Sakha Agric. Res. Station	$SARS_{14}$
Sesbania sesban	Sakha Agric. Res. Station	$SARS_{15}$
Leucaena leucocephala, Lam.	National Res. Center, Dokki	NRC_{16}
Leucaena leucocephala, Lam.	National Res. Center, Dokki	NRC_{17}
Leucaena leucocephale, Lam.	National Res. Center, Dokki	NRC_{18}
Leucaena leucocephala, Lam.	National Res. Center, Dokki	NRC_{19}
Leucaena leucocephala, Lam.	Far. Fac. of Art, Minia Univ.	$FFAMU_{20}$
Leucaena leucocephala, Lam.	Far. Fac. of Art, Minia Univ.	$FFAMU_{21}$
Leucaena leucocephala, Lam.	Far. Fac. of Art, Minia Univ.	$FFAMU_{22}$
Leucaena leucocephala, Lam.	Far. Fac. of Art, Minia Univ.	$FFAMU_{23}$
Leucaena leucocephala, Lam.	Far. Fac. of Art, Minia Univ.	$FFAMU_{24}$
Leucaene leucocephala, Lam.	Ministry of Agric., Minia Govern.	$MAMG_{25}$
Leucaena leucocephala, Lam.	Ministry of Agric., Minia Govern.	$MAMG_{26}$
Leucaena leucocephala, Lam.	Hort. Res. Inst., Agric. Res. Center	HRI_{27}
Leucaena leucocephala, Lam.	Hort. Res. Inst., Agric. Res. Center	HRI_{25}
Leucaena leucocephala, Lam.	Hort. Res. Inst., Agric. Res. Center	HRI_{29}
Leucaena leucocephala, Lam.	Hort. Res. Inst., Agric. Res. Center	HRI_{30}

Far. = Farm.

Table 2: Combination of supplements in minimal agar plates

	1	2	3	4	5
6	Alanine	L-arginie	L-aspartic acid	L-systein	Glycine
7	L-Glutamic acid	L-histidine	L-leucine	L-ysine	DL-methionine
8	L-Phenylalanine	Proline	L-tryptophan	L-tyrosine	DL-valine
9	Adenine	uracil	Biotin	Pyridoxine	Thiamine

All supplements are at a final concentration of 20 μg/ml

liter of YEM medium according to Vincent (1970). YEM with bromo-thymol blue (BTB) was incorporated into agar medium at the rate of 5 ml of 0.5% alcoholic solution per liter. BM_1 medium was used as a basal medium, in this medium phosphates were autoclaved separately. In addition, BM_1-mannitol medium was prepared by adding 1% mannitol (Lafreniere *et al*., 1987). pH was adjusted to 6.7 and 2% agar was added with 1.5-2.0% to solidify the medium. So, BM_2-medium was similar to BM_1, but additionally buffered to pH 7 according to Lafreniere *et al*. (1987).

Mutagenic agents:

Acridines (phase-shift mutations): The effect of acridines would be the specific induction of A-T transversions (C-G to A-T, or A-T to T-A). The mode of action of acridine orange that used in this study was production of frame shifts and it was the product from Sigma Chemical Company, USA. Stock solution of acridine orange was prepared in phosphate buffer (pH 7.5; 10 mmol/L) and filter sterilized according to Pandey and Kashyap (1992).

Ascorbic acid (Vitamin C): L-ascorbic acid, better known as vitamin C, has the simplest chemical structure of all the vitamins. It is a reasonably strong reducing agent. The biochemical and physiological functions of ascorbic acid most likely derive from its reducing properties. It functions as an electron carrier. Loss of one electron due to interaction with oxygen or metal ions leads to semidehydroL-ascorbate, a reactive free radical that can be reduced back to L-ascorbic acid by various enzymes in animals and plants. L-Ascorbic acid, product from Sigma, Co was used in the present study to investigate the mutagenic activity in rhizobia.

Isolation and purification of *Rhizoblum* isolates: The isolates of rhizobia listed in Table 1 were isolated from root nodules of *Sesbania sesban*, *Leucaena leucocephala* (Lam.) according to Chan *et al*. (1988).

Cultural properties, colony morphology and pH reaction: Cultures were prepared by inoculating about 50 ml of yeast extract mannitol liquid medium (YEM) (Vincent, 1970) with a loopful of each of the stored cultures. The flasks were incubated at 28°C with 140 rev min^{-1} for three days. During that time, growth rate was determined (Fitzsimon *et al*., 1992). YEMA medium containing congo red was used to colony morphology characterization. YEMA medium containing 25 mg bromothymol blue (BTB) L^{-1} was used for detecting pH changes. A yellow, blue and green colours

indicated acidity, alkaline and neutral, respectively, according to Norris (1965).

Carbon source utilization: Cells were cultured in liquid mineral medium, collected by centrifugation at 7000 × g for 5 min at 4°C, washed twice with 20 mM sodium phosphate buffer, pH 7.0, containing 1.50 mM NaCl and 3 mM KCl and finally resuspended in mineral medium. After two hours, 20 pL of liquid mineral medium were spotted on the surface of the same solid mineral medium plates and then incubated at 28°C for 4 days. Appropriate carbon source at a final concentration of 1% (w/v) was used according to Milnitsky *et al*. (1997).

Tolerance to salinity of NaCl: Yeast extract agar (YEMA) plates were prepared for NaCl sensitivity. Plates, in three replications, were plated with 0.1 ml of a suitable dilution of an overnight broth culture amended with NaCl concentrations from 3 to 6% (w/v). Plates were incubated at 28°C for three days. Alrplates were visually scored for bacterial growth as separated colonies at zero time and after 24, 36, 72 and 96 hour of incubation. The results were carefully recorded, keeping each part separate.

Growth characteristics: Growth characterization was done with mineral medium for salinity tolerance and VS medium in mutagenicity experiment, as the growth media. Exponential rates were calculated from the bacterial concentrations x_o , x_1, x_2, x_3 and x_4 at the following times, respectively t_o (zero time), t_1 (24 h), t_2 (36 h), t_3 (72 h) and t_4 (96 h) with the equation:

$$\mu = (\log x_1 - \log x_o)/\log_e (t-t_o), \quad MGT = \frac{Ln_2}{\mu}$$

Where, Log_e -= 0.43429, Ln_2 = 0.69314 (Schlegel, 1985).

The log phase was defined as the time between inoculation and establishment of the maximum growth rate (Schlegel, 1985) and was determined graphically by using a plot of log cell number versus time.

Intrinsic antibiotic resistance (IAR): The ability of rhizobial strains to grow on nine different antibiotics at varying concentrations was assessed according to Chan *et al*. (1988). All antibiotics were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Cultures were grown in VS broth to mid-log phase and then diluted to an optical density (OD) of 0.15 or 2×10^7 cells ml^{-1} (Turco *et al*., 1986). Solutions of antibiotics were added to melted VS medium, which had been cooled to about 50°C. The minimum inhibitory concentration was determined by preparing plates with the previous series of antibiotic concentrations, spotting 10 µL of cell suspension (2×10^7 cells ml^{-1}) on a sector of the plate and incubating as described in Cole and Elkan (1973, 1979). The minimum inhibitory concentration is defined as the lowest concentration of an antibiotic which completely inhibits the growth of an organism. The replica plates were incubated until colonies appeared on antibiotic-free plates.

Mutagenic action of acridine and ascorbic acid:
Starvation of bacteria: This method was used before the cells were treated with a mutagenic agents according to Thorne and Williams (1997).

Isolation of auxotrophic mutants: Mutagenesis of *Rhizabium spp.* was carried out by the treatment of the log phase cells grown on BM_1-mannitol, with 60, 120 and 180 µg ml^{-1} for both mutagens used in the present investigation and incubated-for two hours at 28°C on a rotary shaker (160 rev. min^{-1}). The treated cells were washed twice with phosphate buffer saline (PBS; 3 mM phosphate buffer in 0.7% NaCl, pH 6.8), suspended in liquid growth medium (VS) and incubated for 24 h at 28°C on a rotary shaker (160 rev. min^{-1}), to allow segregation and expression of mutations. For enrichment, the cells were washed twice in PBS and resuspended in BM_2 supplemented with the appropriate carbon source and 500 µg ml^{-1} carbenicillin were added. After incubation at 28°C for 24 h on a rotary shaker, the enrichment cycle was repeated. Enriched cultures were washed and plated for single colonies on BM_2 medium modified (Lafreniere *et al*., 1987) by using 0.6 g L^{-1} peptone (Difco) as a nitrogen source and by adding filter sterilized 2, 3, 5-triphenyl tetrazolium chloride (TTC, Sigma) to a final concentration of 25 mg L^{-1}. On this medium, wild type colonies have a good growth and reduce TTC to triphenylformazan (TPF) (pink colonies) (Lenhard, 1956), while mutants show poor growth and TTC is not reduced (white colonies). Mutants were isolated and preserved at -69°C in yeast extract mannitol broth medium containing 10% glycerol (Lafontaine *et al*., 1989). Survival was estimated by comparing with colony counts of untreated control according to Pandey and Kashyap (1992).
The mutation rate, shown previously, was derived from the formula:-

$$m = \log_e 2 \, (M_2-M_1)/(N_2-N_1)$$

Where, M_1 and M2 are the number of mutant clones arising from plates sprayed at time 1 and 2 and N_1 and N_2 are the corresponding total bacterial counts (Beale, 1948). Mutation frequency was calculated according to Eckardt and Haynes (1977).

Identification of biochemical mutants of *Rhirobium*: Twenty possible requirements for single growth factors are tested. Nine plates of minimal medium are each supplemented with different combinations of six growth factors with the arrangement shown in Table 2. A mutant strain with a single biochemical requirement when inoculated on all twelve plates will grow on two of them, one of plates 1-5 and one of plates 6-9 according to (Holliday, 1956).

Statistical analysis: Results were subjected to analysis of variance using the Statistical Analysis System (SAS). When analysis of variance showed significant treatment effects, the Least Significant Difference (LSD) test was applied to make comparisons among the means at 0.05 and 0.01 levels of significance (Steel and Torrie, 1980).

Results and Discussion

Characterization of *Rhizobium* isolates: As shown from the results presented in Table 3 and 4 the isolates displayed a range of colony characteristics. The diameter of colonies of most isolates was 2.2 to 6.8 mm in *Rhizobium* of

Sesbania sesban and 2.5 to 6.2 in *Rhizobium* of *Leucaena leucocephala* and they were circular opaque to translucent. Colonies of a few isolates were greater than 5.2 mm in diameter, creamy white. Although, no quantitative data were collected, no correlation appeared to exist between colony form and isolate origin. The pH reaction of *Sesbania sesban-Rhizobium* isolates was ranged between acidic to neutral, in contrast, all the isolates of *Leucaena leucocephala* showed acidic pH reaction only. The collection of nodules and isolation of bacteria from many locations resulted in a collection of morphologically and biochemically diverse rhizobia that form symbiosis with leguminous trees. Differences among the rhizobial isolates shown here can not be ascribed to the genotype of the tree from which they were collected, because in some cases as much diversity was seen between isolates collected from one tree as between isolates collected from two different trees within one site. These differences were thought to be due to differences among rhizobial strains associated with each nodule (Batzli *et al*., 1992). Variation also has been attributed to soil microsite characteristics such as aeration, nutrient availability, moisture content and competition (Postgate, 1982). These factors may explain variation among isolates in this study.

The results obtained here are in accordance with those reported by Batzli *et al*. (1992), who found that, so much rhizobial diversity was found between the two sites in Green Ridge State Forest (GRSF) in Allegany country, Maryland and even within individual trees in one site, differences in rhizospheric microsite conditioA may be a significant source of variation among strains collected from the two sites. The results indicated that selection of rhizobia strains on the basis of the efficiency of the symbiotic association with leguminous trees is possible. The mean generation time (h) of rhizobial isolates nodulating *Sesbania sesban* and *Leucaena leucocephala* rhizobia ranged from 4.813 to 6.437 and 4.600 to 6.523, respectively. These are in accordance with the results obtained by Batzli *et al*. (1992), who found that the mean generation times of rhizobia *(Robinia pseudoacacia* isolates used in their study was ranged from 3 to 9 h. In addition, the results obtained here are in agreement with the results of the same authors, who found that 186 isolates of rhizobia from nodules of *Robinia pseudoacacia* L. were varied significantly in their resistance to antibiotics and NaCl, their growth on different carbohydrates and their effect on the pH of culture media. Most isolates, of the same authors, showed intermediate antibiotic resistance, the capacity to use numerous carbohydrates and a neutral to acid pH response. The same authors also reported that isolates had greater similarity within sampling locations than among sampling locations.

Effect of salinity on mean generation time (MGTs) of *Rhizobium* sp. related to *Sesbania sesban* and *Leucaena leucocephala*: As shown from the results summarized in Table 5 and 6, the addition of NaCl to YEM broth increased the MGT for all isolates tested. Most of *Rhizobium* strains failed to grow in the highest levels of salt. The salt tolerance of each symbiont of the legume *Rhizobium* symbiosis may differ (Parker *et al*., 1977). Despite a large range in tolerance to salts among species of legumes, there are no agriculture legumes that can be considered to be highly salt tolerant. *Rhizobium* isolates of *Leucaena leucocephala* who showed visible growth on 4% NaCl showed also an increase in MGT. Comparing the sensitivity of the microsymbiont, *Rhizobium*, with that of the legume host will then indicated the relative value of efforts to increase rhizobial tolerance to salts in strain selection programs. The growth of all but some of the rhizobia (*Leucaena*) tested was slowed by the presence of NaCl (Table 6). These results are contrary to those obtained by Pillai and Sen (1973), who showed that the growth rate of *Rhizobium* spp. increased with 1% NaCl added to broth media (EC. 18.0 mS cm^{-1}). The results obtained by Steinborn and Roughley (1975), on the other hand, are in agreement with the results obtained here, who shown that the growth of both *R. trifolii* and *R. meliloti* is slowed by the addition of salt. Large increases in MGT, or growth suppression, was not observed for any of the isolates of *Sesbania sesban* until the concentration of NaCl reached 4%. On the other hand, the growth suppression was observed for all isolates of *Leucaena leucocephala* at the concentration of 5% NaCl. The present results are in agreement with those reported by Hashem *et al*. (1998), who found that the addition of high concentrations of NaCl to YEMB increased the MGT of all *Rhizobium (Leucaena)* strains and some strains failed to grow on 1.5 and 3% NaCl, however some isolates isolated from highly saline soils (Swelim *et al*., 1997) was consistently more tolerant to salt stress than other strain since it alone grew at > 3.0% NaCl. As shown from the results presented here, the addition of high concentrations of NaCl to YEMB increased the MGT of all isolates from two- to five-fold, depending on the strain. This study identifies and characterizes some agronomically important strains of *Sesbania* and *Leucaena* nodulating bacteria from Egypt that are quite salt tolerant and resistant to 3.0% NaCl. The growth rates of these tolerant strains were slowed two- to five-fold by the elevation of salinity from 0 to 3% NaCl. The results obtained here are in accordance with those reported by Saxena *et al*. (1996), who found that salinity-induced changes in the protein profiles in *Rhizobium* sp. exhibited alterations in the expression of as many as 19 proteins, which either showed an enhanced rate of synthesis or a decline in the levels as compared with controls. All these proteins were predominantly of low molecular mass (below 40 kDa) except for one (52 kDa), the difference in the protein profiles was more marked in salt-grown cells as compared with salt-shocked cells.

Carbon source utilization: The results presented in Table 7 and 8 indicate the utilization of monosaccharides (0-glucose, D-fructose and 0-galactose), C3 compounds (glycerol), sugar alcohol (D-mannitol), disaccharides (lactose, maltose and sucrose), pentoses (L-arabinose, Darabinose and xylose) and polysaccharides (dextrin, dextrose and diastase), by different isolates of rhizobialeguminous trees. The rhizobia isolate of *Sesbania sesban* named $FAFMU_3$ did not grow on the mineral medium with inositol, however, the isolate of the same host named $FPMU_7$ did not grow on the mineral medium with L-arabinose, D-arabinose and glycerol as a sole carbon

source. In contrast, all isolates of *Sesbania sesban* rhizobia grew normally on the different carbon sources tested. On the other hand, the isolate of *Leucaena leucocephala* rhizobia named $FFAMU_{21}$ did not grow on the mineral medium with xylose, glycerol and inositol. However, the isolates named $FFAMU_{22}$ and $FFAMU_{23}$ did not grow on the mineral medium (MM) with xylose and glycerol, respectively. In addition, the isolate named $MAMG_{26}$ did not grow on the MM with glycerol and inositol. In contrast, all isolates grew normally on the different carbon sources tested. The biochemical characteristics such as carbon sources utilization were similar to those described for fast-growing rhizobia (Arias *et al*., 1979; Jordan, 1984). The present results are in agreement with those reported by Milnitsky *et al*. (1997), who described some biochemical and genetic characteristics of seven rhizobial isolates that nodulate legume trees, native to Uruguay, they found that carbon utilization by the isolates could be assigned to the fast-growing group of rhizobia. The study of a greater number of isolates from our native legume trees and the use of other techniques will allow us to have a more complete picture about their taxonomy and evolutionary relationship. Rhizobia from woody species have been described as fast, moderate and slow growing (Barnet and Catt, 1991) and many have yet to be assigned to a genus. Sutherland and Sprent (1993) concluded that rhizobia isolated from tree species are highly variable and that it is difficult to assign them to standard genera and species in terms of carbohydrate metabolism, pH change of the growth medium, optimal temperature of growth flagellation and growth rate. The results obtained in this study indicated that the fast-growing isolates of leguminous trees showed excellent growth within 3 days on most of the substrates tested and therefore resembled the fast-growing rhizobia and not the slow-growing species of *Rhizobium*. The slow-growing rhizobia were shown by Graham and Parker (1964) to be more fastidious (Table 8).

Prevalence of intrinsic antibiotic resistance: The minimum inhibitory concentration (WC) of nine antibiotics for wood legume *Rhizobium*, isolated from different locations are shown in Table 9 and 10. The intrinsic antibiotic resistance (IAR) of wood legume rhizobia was extremely high against tetracycline (25 μg ml^{-1}) in the isolates $ARCG_{12}$, $MAMG_{25}$ and $MAMG_{26}$. The lowest minimum inhibitory concentration was occurred with tetracycline (s 25 μg ml^{-1}). Extremely high IAR against ampicillin (150 μg ml^{-1}) was shown in isolates, NRC_{16}, FFAMU24, $MAMG_{25}$ and $FFSMU_i$. These results were comparable with those obtained from temperate fast-growing rhizobia reported by Kremer and Peterson (1982) and Stowers and Eaglesham (1984). Chan *et al*. (1988) reported that intrinsic antibiotic resistance (IAR) method is useful for identifying many *Rhizobium* spp., but its effectiveness for evaluating isolates from *A. sinicus* was inconsistent with certain antibiotic concentration combinations, similar to results by Stein *et al*. (1982), who examined *Rhizobium phaseoli*. This variation of response may be attributed to spontaneous mutations of the organisms (Kingsley and Bohlool, 1983), or to differences in cell load upon inoculation. However, many antibiotic concentration did not provide definite patterns of resistance. Some isolates of rhizobia *Sesbania sesban*, *Leucaena leucocephala*, L. were resistant to high concentration of neomycin (≤ 150 μg ml^{-1}), kanamycin (≤ 150 μg ml^{-1}), streptomycin (≤ 200 μg m^{-1}), spectomycin (≤ 200 μg ml^{-1}), rifampcin (≤ 175 μg ml^{-1}), penicillin (≤ 400 μg ml^{-1}) and chloramphenicol (≤ 250 μg m^{-1}). Soybean and non-soybean rhizobia resistant to high concentration of penicillin and other antibiotics have been reported by Chao (1987), who suggested that the high antibiotic resistance among rhizobia isolated from subtropical-tropical Taiwan soils could be caused by the use of pesticides in the field. In the present study, one isolate of *Sesbania sesban* rhizobia was extremely high against streptomycin (200 μg ml^{-1}). The resistance to streptomycin has proved to be a stable marker with high level of resistance available and little associated loss of symbiotic effectiveness (Levin and Montgomery, 1974). Two isolates of *Leucacena leucocephala* rhizobia were resistant to high concentrations of spectomycin (200 μg ml^{-1}) Spectinomycin resistance in *Rhizobium* is also similar to that of streptomycin and provides a second potential marker for use in ecological studies (Schwinghamer and Dudman, 1973). Chan *et al*. (1988) found that both plasmid visualization and IAR showed similarities with the isolates of *Bradyrhizobium* sp. (*Astragalus sinicus* L). Because antibiotic resistance of an organism is often coded by a plasmid, the smaller plasmid (80 Md) seen in some isolates may carry the gene that codes for tetracycline or neomycin resistance. These were the only isolates which both contained this plasmid and showed resistance to tetracycline and neomycin. $MAMG_{25}$, ampicillin resistant culture (≤ 150 μg ml^{-1}) was resistant to the same concentration of both neomycin and kanamycin. HRI_{27}, also showed the same trend for both ampicillin (≤ 150 μg ml^{-1}). Cultures of $FAFMU_3$ and $FAFMU_5$ resistant to neomycin (≤ 150 μg ml^{-1}) were resistant to penicillin (≤ 400 μg ml^{-1}), $FAFMU_6$ also appeared a similar nature for both penicillin (≤ 400 μg ml^{-1}) and chloramphenicol (≤ 300 μg ml^{-1}). However, $ARCG_{10}$ streptomycin resistant culture (≤ 200 μg ml^{-1}) was resistant to penicillin (≤ 400 μg m^{-1}). It is reported that the reduction in symbiotic effectiveness of 15 of the 59 resistant strains of *Bradyrhizobium* for desmodium intortum is in agreement with Bromfield and Jones (1979) observation that antibiotic resistance can be associated with decreased effectiveness. However, their results offer little support for a general association between loss of effectiveness and resistance to particular groups of antibiotics, including streptomycin and rifampicin (Pankhurst, 1977).

Mutation frequencies of acridine orange in *Rhizoblum* of legume trees: The results summarized in Table 11-14 demonstrate a dose-response for decreasing cell survival at levels that are not excessively toxic to the bacteria at both pre-incubation and plate incorporation assays. The standard assay without pre-incubation involves adding, in order, the test compound, the bacterial tester strain, to soft agar, which is then briefly mixed and poured directly onto the BM_2 agar plate. This assay requires somewhat less time and fewer manipulations than the modification with pre-incubation, but is somewhat less sensitive for some mutagens. The standard assay with pre-incubation was quite toxic to the bacteria than plate incorporation test. This are in accordance with Adams (1959), who reported that acridines inhibit bacteriophage development at concentrations below those inhibit the bacterial host.

Mutagenicity data for acridine orange appeared here revealed that in all cases the addition of plate incorporation resulted in equal or greater sensitivity than the standard assay of pre-incubation. A reproducible dose-response with an increase in the number of auxotrophic mutants as shown

Table 3: Some phenotypic characteristics of *Rhizobium* isolates nodulating *Sesbania sesban*

Isolates	pH reaction	Growth at pH range	Mean generation time (h)	Opacity	Colony morphology Viscosity	Size (mm)
FFSMU-1	Acidic	5-11	6.090	Opaque	High	2.6
FAFMU-2	Acidic	5-10	5.363	Opaque	High	5.3
FAFMU-3	Acidic	5-10	5.487	Opaque	High	5.4
FAFMU-4	Acidic	5-10	5.940	Opaque	Meduim	4.8
FAFMU-5	Acidic	5-11	5.277	Translucent	Low	4.4
FAFMU-6	Acidic	5-10	6.437	Translucent	Low	4.2
FPMU-7	Acidic	4-10	6.200	Semi-trans lucent	Low	5.2
FFAMU-8	Acidic	5-10	5.480	Semi-trans lucent	Low	2.2
ARCG-9	Neutral	4-10	6.120	Semi-trans lucent	Low	6.8
ARCG-10	Acidic	5-10	6.373	Opaque	Low	4.1
ARCG-11	Acidic	5-11	5.387	Translucent	High	4.6
ARCG-12	Acidic	5-10	5.710	Translucent	Medium	4.8
SARS-13	Neutral	5-11	4.813	Translucent	High	3.6
SARS-14	Neutral	5-11	5.097	Translucent	Low	4.4
SARS-15	Acidic	4-10	5.170	Translucent	Medium	3.0

Table 4: Some phenotypic characteristics of *Rhizobium* isolates nodulating *Leucaena leucocephala*

Isolates	pH reaction	Growth at pH range	Mean generation time (h)	Opacity	Colony morphology Viscosity	Size (mm)
NRC-16	Acidic	5-10	5.047	Translucent	Low	4.10
NRC-17	Acidic	5-10	5.357	Translucent	Low	2.50
NRC-18	Acidic	4-10	6.397	Translucent	Low	5.00
NRC-19	Acidic	5-11	5.287	Semi-Trans- lucent	Low	5.20
FFAMU-20	Acidic	5-10	5.043	Semi-Trans- lucent	High	5.00
FFAMU-21	Acidic	5-10	4.600	Semi-Trans- lucent	Low	4.40
FFAMU-22	Acidic	5-10	5.680	Semi-Trans- lucent	High	5.20
FFAMU-23	Acidic	4-11	5.267	Opaque	Medium	4.30
FFAMU-24	Acidic	4-10	5.040	Opaque	Medium	4.10
MAMG-25	Acidic	5-10	6.523	Opaque	High	5.20
MAMG-26	Acidic	5-10	✓5.457	Opaque	High	6.20
HRI-27	Acidic	5-10	5.097	Translucent	High	5.90
HRI-28	Acidic	5-11	5.570	Translucent	High	6.90
HRI-29	Acidic	5-11	6.053	Translucent	Medium	5.80
HRI-30	Acidic	4-10	5.340	Translucent	Medium	3.80

Table 5: Mean generation time (h) of *Sesbania sesban*-rhizobia under salt stress at varying degrees

	Concentration of NaCl (%)					
Isolates	1	3	4	5	6	Relative Sensitivity
FFSMU-1	6.090	9.366	NG	NG	NG	1.54
FAFMU-2	5.363	12.606	NG	NG	NG	2.35
FAFMU-3	5.446	9.346	NG	NG	NG	1.71
FAFMU-4	5.940	8.346	NG	NG	NG	1.41
FAFMU-5	5.276	7.380	NG	NG	NG	1.40
FAFMU-6	6.436	9.86	NG	NG	NG	1.53
FPMU-7	6.200	13.33	NG	NG	NG	2.15
FFAMU-8	5.480	17.21	NG	NG	NG	3.14
ARCG-9	6.120	12.05	NG	NG	NG	1.97
ARCG-10	6.373	12.70	NG	NG	NG	1.99
ARCG-11	5.386	14.446	NG	NG	NG	2.68
ARCG-12	5.710	13.606	NG	NG	NG	2.38
SARS-13	4.813	NG	NG	NG	NG	1.00
SARS-14	5.096	NG	NG	NG	NG	1.00
SARS-15	5.170	NG	NG	NG	NG	1.00
F-test	**	**				**
L.S.D.	0.05	0.698	2.287			0.38
	0.01	0.940	3.079			0.52

NG = No growth

here over the spontaneous value is evidence for mutagenicity.

Acridine hypersensitivity has also been found in T4 phage with mutations in a variety of genes, many of which are DNA synthesis and repair genes, implying that some inhibitory effects of acridines act at this level (Woodworth and Kreuzer, 1996). An alternative view, but forward by Brenner *et al*. (1961), is that acridine mutations and the majority of spontaneous mutations are not caused by base-pair substitutions at all, but by the deletion or addition of a base-pair. Obviously such mutations could not be reverted by any agent whose effect was simply to replace one base-pair by another, but only by the restoration of a deleted base or the removal of an extra one.

Mutaganic activity of ascorbic acid: The data are presented in Table 15-18 show that ascorbic acid appeared genotoxic hazards for *Rhizobium.* Data are also

Table 6: Mean generation time (h) of *Leucaene leucocephala*-rhizobia under salt stress at varying degrees

	Concentration of NaCl (%)					
Isolates	1	3	4	5	6	Relative Sensitivity
NRC-16	5.046	12.936	40.336	NG	NG	7.99
NRC-17	5.336	9.863	59.303	NG	NG	11.07
NRC-18	6.396	NG	NG	NG	NG	1.00
NRC-19	5.286	11.590	NG	NG	NG	2.19
FFAMU-20	5.073	14.833	NG	NG	NG	2.923
FFAMU-21	4.533	20.300	NG	NG	NG	4.02
FFAMU-22	5.680	61.243	NG	NG	NG	10.43
FFAMU-23	5.866	20.300	32.55	NG	NG	5.54
FFAMU-24	5.040	19.240	26.75	NG	NG	5.30
MAMG-25	6.523	27.543	NG	NG	NG	4.22
MAMG-26	5.456	29.426	NG	NG	NG	5.39
HRI-27	5.096	30.493	NG	NG	NG	5.24
HRI-28	5.570	33.160	NG	NG	NG	5.95
HRI-29	6.053	45.493	NG	NG	NG	7.52
HRI-30	5.340	40.190	NG	NG	NG	6.86
F-test	**	**	**			**
L.S,D.	0.05	0.522	8.100	6.574		1.89
	0.01	0.702	10.906	8.850		2.55

NG = No growth

Table 7: Phenotypic evaluation of *Rhizobium* sp. isolated from *Sesbania sesban* nodules

Isolates	FFSMU	FAFMU	FAFMU	FAFMU	FAFMU	FAFMU	FAFMU	FAFMU	ARCG	ARCG	ARCG	ARCG	SARS	SARS	SARS
Carbon sources	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pantose:															
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D- arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hexoses:															
D-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Disaccharide:															
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sugar alcohol:															
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C3 compound:															
Glycerol	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Polysaccharide:															
Dextrin	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Diastase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+

+ = Visible growth - = Invisible growth

Table 8: Phenotypic evaluation of *Rhizobium* sp. isolated from the nodules of *Leucaena leucocephale*, Lam

isolates	NRC-	NRC-	NRC-	NRC-	FFAMU-	FFAMU-	FFAMU-	FFAMU-	FFAMU-	MAMG-	MAMG-	HRI-	HRI-	HRI-	HRI-
Carbon sources	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Pantose:															
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0-arabinose	+	+	+	+	+	+	. +	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+		
Hexoses:															
0-fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
0-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Disaccharide:															
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sugar alcohol:															
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C3 compound:															
Glycerol	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+
Polysaccharide:															
Dextrin	+	-	-	-	+	+	+	-	-	-	-	-	-	-	+
Dextrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Diastase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = Visible growth - = Invisible growth

Table 9: Minimum inhibitory concentration (µg ml^{-1}) of nine antibiotics against *Rhizobium* of *Sesbenia sesben*

Isolates	Tetracycline	Ampicillin	Neomycin	Kanamycin	Spectomycin	Rifampcin	Pencillin-G	Chlora-mphenicol	Streptomycin
FFAMU-1	15	150	75	100	100	50	275	100	150
FFAMU-2	15	100	75	45	100	100	460	75	75
FFAMU-3	5	75	75	50	150	75	350	75	125
FFAMU-4	20	100	150	150	175	75	400	125	100
FFAMU-5	10	125	150	75	100	50	400	175	150
FFAMU-6	5	75	75	75	100	50	400	300	150
FFAMU-7	5	75	150	100	175	75	275	150	150
FFAMU-8	15	75	75	100	100	100	200	50	100
ARCG-9	20	75	75	125	100	75	125	100	150
ARCG-10	10	125	100	75	100	50	400	275	200
ARCG-11	10	100	50	100	100	50	150	225	75
ARCG-12	25	75	150	125	100	50	300	225	125
SARS-13	10	75	50	50	50	75	300	125	100
SARS-14	15	50	100	100	100	75	300	250	175
SARS-15	15	75	150	125	75	75	325	225	150

Table 10: Minimum inhibitory concentration (µg ml^{-1}) of nine antibiotics against *Rhizobium* of *Leucaene leucocephale*

Isolates	Tetracycline	Ampicillin	Neomycin	Kanamycin	Spectomycin	Rifampcin	Pencillin-G	Chlora-mphanicol	Streptomycin
NRC-16	15	150	100	50	100	75	300	125	125
NRC-17	20	15	75	75	100	100	300	150	150
NRC-18	10	75	125	50	100	100	125	150	125
NRC-19	10	100	125	75	100	150	375	75	125
FFAMU-20	10	50	125	75	100	50	350	300	75
FFAMU-21	15	50	50	50	100	75	325	225	100
FFAMU-22	20	50	125	75	175	75	175	100	100
FFAMU-23	10	75	100	50	125	75	250	100	100
FFAMU-24	20	150	125	100	100	75	250	100	75
MAMG-25	25	150	150	150	125	75	175	150	100
MAMG-26	15	100	150	125	125	125	200	100	125
HRI-27	25	100	100	100	200	150	400	100	125
HRI-28	10	75	75	100	175	75	200	125	75
HRI-29	10	25	75	100	100	125	325	125	75
HRI-30	10	150	150	75	200	125	350	150	125

Table 11: Mutagenicity of acridine orange towards FFAMU-8 strain of *Sesbanis sesban* rhizobia

Concentration µg ml^{-1}		Preincubation					Plate-incorporation					Mutation Rate
		1	2	3	4	5	1	2	3	4	5	
0.00		6.6	100.0	0.0	0.0	0.0	55.0	100.00	0.003	0.006	0.40	0.00
60		4.3	24.66	0.28	6.49	6.43	25.0	45.45	1.20	4.80	4.20	3.07
120		2.7	48.36	0.50	20.93	20.54	5.40	9.81	1.48	27.47	26.86	18.31
180		1.5	30.71	0.23	15.0	15.00	2.30	4.30	0.70	24.73	29.13	41.07
F-test		**	**	**	**	**	**	**	**	**	**	**
L.S.D.	0.05	0.53	8.70	0.13	9.09	8.44	1.20	1.89	0.17	6.14	6.16	11.32
	0.01	80.00	13.17	0.15	13.7	13.53	1.82	2.87	0.26	9.29	9.32	17.13

**Significant at 0.01 of probability level

Table 12: Mutagenicity of acridine orange towards ARCG-10 strain of *Sesbania sachem* rhizobia

Concentration µg ml^{-1}		Preincubation					Plate-incorporation					Mutation Rate
		1	2	3	4	5	1	2	3	4	5	
0.0		4.40	100.0	0.01	0.02	0.25	54.00	100.0	0.01	0.024	0.24	0.01
60		2.16	49.29	0.28	13.16	12.40	35.00	64.81	1.80	5.14	4.34	3.20
120		1.33	30.30	0.20	15.48	14.70	4.63	8.57	1.30	28.18	27.93	2.30
180		1.16	26.51	0.23	20.19	19.43	2.60	4.93	0.70	26.20	25.96	21.54
F-test		**	**	**	**	**	**	**	**	**		
L.S.D.	0.05	0.77	4.08	0.13	210.30	10.45	1.51	1.69	0.33	7.61	7.60	11.82
	0.01	1.17	6.18	0.19	15.60	15.83	2.27	2.56	0.50	11.52	11.52	17.90

*Significant at 0.01 of probability level

Table 13: Mutagenicity of acridine orange towards NRC-19 strain of *Laucaana leucocephale* rhizobia

Concentration µg ml^{-1}		Preincubation					Plate-incorporation					Mutation Rate
		1	2	3	4	5	1	2	3	4	5	
0.0		5.06	100.0	0.01	0.19	0.06	42.66	100.0	0.03	0.08	0.25	0.05
60		3.13	61.79	0.20	6.29	6.09	28.00	65.60	0.63	2.23	2.23	1.19
120		2.43	47.98	0.66	28.04	27.84	6.63	15.54	1.56	23.62	23.62	14.69
180		1.40	27.61	0.36	26.28	27.67	2.70	6.09	0.86	32.07	32.07	26.70
F-test		**	**	**	**	**	**	**	**	**	**	**
L.S.D.	0.05	0.89	9.48	0.12	8.56	8.22	2.80	6.37	0.32	8.55	2.88	4.82
	0.01	1.36	14.35	0.67	12.95	12.44	4.24	9.65	0.49	12.95	4.36	7.31

*Significant at 0.01 of probability level

Table 14: Mutagenicity of acridine orange towards HRI-27 strain of *Leucaena leucocephala* rhizobia

Concentration µg ml^{-1}		Preincubation					Plate-incorporation					Mutation Rate
		1	2	3	4	5	1	2	3	4	5	
0.0		4.66	100.0	0.03	0.073	0.05	55.67	100	0.03	0.05	0.01	0.04
60		3.50	74.99	0.50	13.26	13.14	12.33	22.45	1.26	10.15	10.09	6.19
120		2.40	51.43	0.63	26.29	26.22	6.63	10.11	1.60	28.56	29.23	23.50
180		1.030	22.14	0.23	23.33	23.26	2.10	3.77	0.70	33.31	33.25	30.66
F-test		**	**	**	**	**	**	**	**	**	**	**
L.S.D.	0.05	0.49	7.53	0.22	11.46	11.45	1.97	1.26	0.13	5.74	5.48	11.46
	0.01	0.74	11.40	0.33	17.36	17.34	2.98	1.90	0.19	2.69	8.76	17.36

1 = Total No. of colonies ml$^{-1} \times 10^6$ = frequency $\times$ 1 0^{-6} =2 = Survival (%) 3 = Total No. Of mutant ml$^{-1} \times 10^6$ + 4 = Mutagenicity (%) + 5 + Mutation

**Significant at 0.01 of probability level

Table 15: Mutagenicity of ascorbic acid towards FFAMU-8 strain of *Sesbania sesban* rhizobia

Concentration µg ml^{-1}		Preincubation					Plate-incorporation					Mutation Rate
		1	2	3	4	5	1	2	3	4	5	
0.0		24.00	100.0	0.10	4.31	0.03	143.30	100.0	2.00	1.375	0.003	0.05
60		9.60	40.27	3.66	38.42	0.34	34.30	2.40	17.00	50.31	0.49	0.29
120		2.73	11.38	1.53	60.60	0.56	1.40	10.01	3.66	26.42	0.25	0.13
180		1.23	5.136	1.23	46.52	0.59	12.53	8.62	4.00	32.63	0.31	0.22
F-test		**	**	**	**	**	**	**	**	**	**	**
L.S.D.	0.05	3.29	12.12	0.93	37.82	0.33	16.87	6.27	4.72	19.71	0.20	0.11
	0.01	4.99	18.35	1.41	57.27	0.42	25.53	9.49	7.15	29.84	0.30	0.17

**Significant at 0.01 of probability level

Table 16: Mutagenicity of ascorbic acid towards ARCG-10 strain of *Sesbania sesban* rhizobia

Concentration µg ml^{-1}		Preincubation					Plate-incorporation					Mutation Rate
		1	2	3	4	5	1	2	3	4	5	
0.0		37.00	100.0	2.66	7.59	0.02	176.0	100.0	8.00	2.79	0.01	0.03
60		23.00	62.15	12.33	67.36	0.60	123.0	69.78	15.33	13.43	0.11	0.02
120		3.60	9.90	3.23	87.70	0.80	21.66	12.28	12.33	57.06	0.54	0.35
180		1.53	4.14	1.53	66.10	0.59	35.33	19.94	16.33	46.85	0.44	0.41
F-test		**	**	**	**	**	**	**	**	**	**	**
L.S.D.	0.05	8.70	9.41	3.16	31.97	0.32	34.87	18.14		14.56	0.15	0.23
	0.01	13.18	14.25	4.78	48.42	0.49	52.80	27.47		22.05	0.22	0.34

**Significant at 0.01 of probability level

Table 17: Mutagenicity of ascorbic acid towards NRC-19 strain of *Leucaena leucocephala* rhizobia

Concentration µg ml^{-1}		Preincubation					Plate-incorporation					Mutation Rate
		1	2	3	4	5	1	2	3	4	5	
0.0		33.66	100	0.00	0.00	0.00	133.33	100.0	0.00	0.00	0.00	0.00
60		11.00	32.67	6.00	53.70	0.53	25.66	19.25	3.33	11.42	0.53	0.38
120		5.63	16.72	3.53	61.54	0.61	17.66	13.25	12.33	70.77	0.70	0.53
180		1.47	5.24	1.48	82.18	0.81	2.66	7.25	5.66	59.84	0.59	0.74
F-test		**	**	**	**	**	**	**	**	**	**	**
L.S.D.	0.05	4.28	6.16	2.05	15.18	0.15	18.83	5.18	1.49	14.84	0.15	0.19
	0.01	0.48	9.33	3.11	22.98	0.23	28.51	7.84	2.26	22.23	0.23	0.28

* Significant at 0.01 of probability level

Table 18: The mutagenicity of ascorbic acid towards 1-1131-27 strain of *Leucaena leucocephala rhizobia*

Concentration µg ml^{-1}		Preincubation					Plate-incorporation					Mutation Rate
		1	2	3	4	5	1	2	3	4	5	
0.0		18.00	100.0	0.0.0	0.00	0.00	62.66	100.0	0.00	0.00	0.00	0.00
60		13.00	72.21	6.33	49.35	0.49	45.33	7.22	25.66	56.32	0.44	0.42
120		3.20	17.77	2.20	69.42	0.69	46.33	7.38	31.66	68.79	0.68	0.48
180		0.86	4.81	0.53	61.11	0.60	36.33	5.79	21.00	59.91	0.59	0.42
F-test		**	**	**	**	**	**	**	**	**	**	**
L.S.D.	0.05	1.70	5.98	2.92	25.85	0.26	13.65	1.08	6.74	22.28	0.19	0.14
	0.01	2.58	9.06	4.42	39.15	0.39	20.67	1.64	10.21	33.74	0.29	0.22

1 = Total No. of colonies ml$^{-1} \times 10^7$ 2 = Survival (%) 3 = Total No. Of mutant ml$^{-1} \times 10^7$, 4 = Mutagenicity (%), 5 = Mutation frequency $\times 10^{-7}$ ** Significant at 0.01 of probability level

indicating that ascorbic acid demonstrate a dose-response for both cell survival and mutagenicity at levels that are notexcessively toxic to the bacteria. The standard assay without pre-incubation (plate incorporation) involves adding, in order, the test compound and the bacterial tester strain to agar plates. The mutagenic activity of ascorbic

Table 19: Identification of biochemical mutants of *Rhizobium* using nine plates of minimal medium each supplemented with different combinations of growth factors

	Plate No.								
	1	2	3	4	5	6	7	8	9
acr-1 FFAMU-8	-	-	-	+	-	+	-	-	-
acr-2 FFAMU-8	+	-	-	-	-	+	-	-	-
acr-3 FFAMU-8	-	-	+	-	-	-	-	+	-
acr-4 FFAMU-8	+	+	+	+	+	+	+	+	+
asc-1 FFAMU-8	-	-	+	-	-	-	-	+	-
asc-2 FFAMU-8	-	-	-	-	+	-	+	-	-
asc-3 FFAMU-8	-	+	-	-	-	+	-	-	-
asc-4 FFAMU-8	-	-	+	-	-	-	+	-	-
acr-1 ARCG-10	+	-	-	-	-	-	-	+	-
acr-2 ARCG-10	-	-	-	-	+	+	-	-	-
acr-3 ARCG-10	-	-	-	-	-	-	-	-	-
acr-4 ARCG-10	-	+	-	-	-	+	-	-	-
asc-1 ARCG-10	-	-	-	-	+	-	+	-	-
asc-2 ARCG-10	+	-	-	-	-	-	+	-	-
asc-3 ARCG-10	-	-	-	+	-	-	-	-	+
asc-4 ARCG-10	-	-	-	+	-	-	-	-	-
acr-1-NRC-19	-	-	+	-	-	-	-	+	-
acr-2-NRC-19	-	-	-	-	+	-	-	+	-
acr-3-NRC-19	-	+	-	-	-	+	-	-	-
acr-4-NRC-19	+	-	-	-	-	-	+	-	-
asc-1-NRC-19	-	-	+	-	-	-	-	+	-
asc-2-NRC-19	+	-	-	-	-	-	-	-	-
asc-3-NRC-19	+	-	-	-	-	-	-	-	+
asc-4-NRC-19	-	-	-	-	-	-	-	-	-
acr-1-HRI-27	-	-	-	-	+	-	-	+	+
acr-2-HRI-27	+	+	+	+	+	+	+	+	-
acr-3-HRI-27	-	-	-	+	-	-	+	-	-
acr-4-HRI-27	-	-	-	-	+	-	-	+	-
asc-1-HRI-27	-	-	-	-	-	-	-	-	-
asc-2-HRI-27	-	-	+	-	-	+	-	-	-
asc-3-HRI-27	-	-	-	-	-	-	-	-	-
asc-4-HRI-27	+	-	-	-	-	-	+	-	-

-+ = Positive growth -=Negative growth
acr and asc Auxotrophic mutant derived from acridine and ascorbic acid treatments, respectively

Table 20: Genotype identification of *Rhizobium* (*Sesbania sesban*) auxotrophic mutants

Mutants	Source and/or reference	Mutagen	Genotype
acr-1-FFAMU-8	FFAMU-8	Acridine orange	sys$^-$
acr-2-FFAMU-8	FFAMU-8	Acridine orange	*ala*$^-$
acr-3-FFAMU-8	FFAMU-8	Acridine orange	*trp*$^-$
acr-4-FFAMU-8	FFAMU-8	Acridine orange	Revertant to wild type
asc-1-FFAMU-8	FFAMU-8	Ascorbic acid	*trp*$^-$
asc-2-FFAMU-8	FFAMU-8	Ascorbic acid	*met*
asc-3-FFAMU-8	FFAMU-8	Ascorbic acid	*arg*$^-$
asc-4-FFAMU-8	FFAMU-8	Ascorbic acid	*leu*$^-$
acr-1-ARCG-10	ARCG-10	Acridine orange	*phe*
acr-2-ARCG-10	ARCG-10	Acridine orange	*gly*
acr-3-ARCG-10	ARCG-10	Acridine orange	Other requirements
acr-4-ARCG-10	ARCG-10	Acridine orange	*arg*$^-$
asc-1-ARCG-10	ARCG-10	Ascorbic acid	*met"*
asc-2-ARCG-10	ARCG-10	Ascorbic acid	*glu*$^-$
asc-3-ARCG-10	ARCG-10	Ascorbic acid	*pyrid*$^-$
asc-4-ARCG-10	ARCG-10	Ascorbic acid	sys$^-$

acr = Acridine orange = asc Ascorbic acid

Table 21: Genotype identification of *Rhizobium* (*Leucaena leucocephala*) auxotrophic mutants

Mutants	Source and/or reference	Mutagen	Genotype
acr-1-NRC-19	NRC-19	Acridine orange	*trp*$^-$
acr-2-NRC-19	NRC-19	Acridine orange	*vat*
acr-3-NRC-19	NRC-19	Acridine orange	*arg*$^-$
acr-4-NRC-19	NRC-19	Acridine orange	*glu*$^{--}$
asc-1-NRC-19	NRC-19	Ascorbic acid	*trp*
asc-2-NRC-19	NRC-19	Ascorbic acid	*ade*
asc-3-NRC-19	NRC-19	Ascorbic acid	Revertant to wild type
asc-4-NRC-19	NRC-19	Ascorbic acid	*vat*
acr-1-HRI-27	HRI-27	Acridine orange	Revertant to wild type /ys$^-$
acr-2-HRI-27	HRI-27	Acridine orange	*vat*
acr-3-HRI-27	HRI-27	Acridine orange	Other requirements
acr-4-HRI-27	HRI-27	Acridine orange	*asp*$^-$
asc-1-HRI-27	HRI-27	Ascorbic acid	*glu*$^-$
asc-2-HRI-27	HRI-27	Ascorbic acid	Other requirements
asc-3-HRI-27	HRI-27	Ascorbic acid	*glu*$^-$
asc-4-HRI-27	HRI-27	Ascorbic acid	

acr = Acridine orange, asc = Ascorbic acid

acid in all isolates tested resulted in equal or greater sensitivity in the addition of pre-incubation than without it (plate incorporation) at most concentrations used. The standard assay without pre-incubation is preferable for generating a dose-response curve and for general testing (Levin *et al*., 1984). Recent reports have indicated that ascorbic acid, at high concentrations, may be mutagenic (Galloway and Painter, 1979) and cytotoxic (Stich *et al*., 1979) in the *in vitro* mutagensis bioassays. This effect was initially attributed to the generation of hydrogen peroxide (H_2O_2) and/or hydroxyl free radicals (F101, but this does not seem to be clear.

These results reported here also show a dose-response for mutagenicity ratio in all rhizobial isolates tested. These are in agreement with Stich *et al*. (1976), who reported that oxidation products of ascorbic acid are mutagenic for microbial and mammalian cells; ascorbic acid in the presence of oxygen converts covalently-closed circular DNA to open circular DNA molecules, causes single strand breaks in DNA (Bode, 1967). The results obtained in this study are also in harmony with those found by Amabile-Cuevas *et al*. (1991), who reported that treatment of *Staphylococcus aureus* with 1 mM ascorbic acid for 6 h induced an increased loss of resistance markers in 4 of 6 strains tested and agarose gel electrophoresis showed the disappearance of plasmid DNA in ascorbate-induced susceptible colonies. In addition, the presence of ascorbate induced a 50-75% decrease in minimal inhibitory concentrations of different antibiotics for resistant strains of *Staphylococcus aureus*.

Identification of biochemical mutants: As shown from the results presented in Table 19, nine plates of minimal medium are each supplemented with different combinations of growth-factors with the arrangement shown in the related (Table 2) presented in Materials and Methods. The number of requirements tested can be varied by altering the number of plates and the arrangement of growth-factors among them. Most of biochemical mutants as shown here in with a single biochemical requirement, because when inoculated on nine plates will grow on two of them, one of plates 1-5 and one of plates 6-9. Although, some of biochemical mutants such as acr-4 FFAMU-8 and acr-2-HRI27 were reverted to wild type, because of their ability to grow on all of minimal medium plates. In contrast, some others such as acr-3 ARCG-10, asc-4-NRC-19, asc-1-HRI27 and asc-3-HRI-27 failed to grow on any of minimal medium plates, indicating that these mutants have another requirements than that are listed in the nine plates recorded in this study. Mutants requiring for example, cysteine for growth, will only grow on plates 4 and 6. Mutants with alternative requirements will grow on more than two plates. When large numbers of mutants are to be identified, this technique is more efficient than previous methods in which the field of each is progressively reduced. With one or a few mutants auxanographic techniques are preferable. The present method for the identification of biochemical mutants of *Rhizobium* has been carried out according to Holliday (1956).

Leading to the results presented in Table 19-21 summarizes the identification of biochemical mutants induced. In this respect, once a mutant has been isolated, it is frequently desirable to characterize the nature of the genetic defect by further biochemical or genetic analyses. For example, a nutritional requirement can result from mutations in several genes. Thus, an auxotrophic mutant unable to synthesize a specific amino acid, for example, may be blocked in any one of the several sequential enzymatic reactions in that synthetic pathway.

The results obtained in this study are in agreement with those reported by Kerppola and Kahn (1988), who found that mutants in ornithine transcarbamylase, argininosuccinate synthetase or serine-glycine biosynthesis formed nitrogen-fixing (Fix^+) nodules on the roots of alfalfa *(Medicago sativa),* as well as prototrophic revertants were always fix*. Malek (1974) found that most of ineffective auxotrophic mutants of *Rhizobium meliloti* required histidine, histidine + cysteine, cysteine, arginine or casamino acid + yeast extract showed resistance to five tested phages. The finding of Malek (1974) support observation of Kleczkowska (1965) that mutation to phage resistance in *Rhizobium* frequently causes their ineffectiveness. On the other hand, Atkins and Hayes (1972) tested four Wage resistant mutants of *R. trifolii* and concluded that these mutations were concerned with modification of constitution or organization of the polymers of the cell wall which caused degradation of lipopolisaccharides, known as phage receptors in gram-negative bacteria. Abdel-Wahab (1977) reported that single auxtrophic mutants induced from the effective *Rhizobium trifolii* WTG, requiring *His, Lys^-, Leu^-, Ade^- or Ura^-* were ineffective. However, these requiring *Arg^-, $Meth^-$* or *Trp*-more effective in nitrogen fixation as the original WTG. Also, double auxtrophic mutants, *Arg His, Leu^-, Meth, Lys, $Tryp^-$, Leu^- Ade^-* and *Lys^- Ura^-* were ineffective in nitrogen fixation.

To summarize, the results reported here show that when using the "treated-and-plate" test in mutant experiments, one has to control the physiological state of the cells very carefully. This is especially important when the bacteria are treated in non-growth medium with simple acridine and ascorbic acid which do not damage DNA, but acridine induce farmeshift mutations only when present during replication. On the other hand, ascorbic acid is mutagenic and cytotoxic probably through the generation of hydrogen peroxide if added to bacteria in a medium prepared with sterile tap water.

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