FACTORS AFFECTING RHIZOBIUM LEGUME SPECIFICITY IN RHIZOBIUM TRIFOLII

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University for the Degree of

Master of Science

by

Shellemiah Okoth Keya

June, 1970

BIOGRAPHICAL SKETCH

Shellemiah Okoth Keya was born on September 22, 1941 at Angino, Kenya. He attended Ringa and Wang'apala Primary Schools, Kisii and Kakamega High Schools. The author taught and became Headmaster of President Kennedy High School, Nairobi before joining the University of East Africa at Makerere in 1965.

In March 1968, he graduated from Makerere in B. Sc. (Agric.) with honors, winning Uganda Company Exhibition and Muljibhai Madhvani Prizes in 1967 and 1968 respectively.

He entered the Graduate School at Cornell University, Ithaca, New York in September 1968 as a candidate for the Master of Science degree, majoring in Soil Science and minoring in Microbiology.

ACKNOWLEDGMENTS

The author is grateful to Dr. Martin Alexander for his guidance, enthusiasm and suggestions during this study training. Thanks are due to Dr. H. B. Naylor, who served on his academic committee.

Assistance of the following people is highly appreciated: Dr. H. A. MacDonald, for identifying *Trifolium* plants; Dr. Jacob Schiffman and Mr. D. I. deSouza, for supplying *Trifolium* seeds; Dr. David Hubbell and Mr. A. J. Francis, for discussion and supplying some *Rhizobium* cultures; And Mr. G. H. Kiruwa, for statistical analysis.

Thanks is also expressed to United States Agency for International Development for a fellowship which permitted this study training.

Finally, particular thanks are due to Mrs. Joyce J. Gombe, for typing this manuscript.

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INTRODUCTION

Interest in the genus Rhizobium lies, above all, in its ability to form a symbiotic association with legumes in which nitrogen is fixed, this latter fact being of great agronomic importance. When free-living in soils or in the rhizosphere of plants, there is nothing of great interest in the effect of these facultative symbionts. The contentious nature of the subject is brought about by the fact that legume host-macrosymbiont and bacteria-microsymbiont is so variable; the techniques though elegant have not got near to the solution of some of the problems. Despite the vast amount of literature in Rhizobium field, knowledge has not yet reached the stage at which the critical factors governing the bacterium-host interaction can be recognized.

Root-nodule bacteria are known to infect the roots of specific leguminous host plants. A nitrogen fixing symbiosis is usually, but does not always establish in the nodules resulting from the infections. Whereas it is established that most rhizobia are specific for legume species, many will nodulate outside their hosts. (2), (38). Since attempts to classify rhizobia on the basis of their morphological, nutritional or physiological characteristics have not met with much success, the unsatisfactory system of classification based on host specificity still holds. Symbiotic specificity is determined at the infective or non infective level, i.e. ability of rhizobia to infect and form nodule in a homologous host.

Whereas the physical events of the nodulation process are well documented, the biochemical factor(s) determining the unique specificity of a rhizobial strain for a particular host plant or range of host plants is entirely unknown. Li and Hubbell (25), in their investigation of the basis for determination of nodulating specificity in Rhizobium-clover associations, concluded that in rhizobia-clover combinations which nodulate via infection threads, specificity is determined at or before infection thread initiation.

Observations of other workers that rhizobia produce a strain-specific substance affecting growth and morphology of legume root hairs were also confirmed by results of Li and Hubbell (25).

The mechanism of invasion advanced by Fahraeus and Ljunggren (13), by Rhizobium spp. of their host plants has been widely quoted; e.g., by Nutman (40), Vincent (54) and Munns (35). Polygalacturonase has been postulated by Fahraeus and Ljunggren (13), Munns (35) as being involved in the invasion of the host legume by Rhizobium spp. Involvement of polygalacturonase has now been confirmed by Munns (37). However, Thomas and Elkan (52) found no greater levels of enzyme activity from effective plant-bacteria associations than from either ineffective associations or uninoculated control plants. They concluded that polygalacturonase does not play a major role in the invasion of Glycine max by Rhizobium japonicum. This controversial hypothesis is still being questioned because evidence for polygalacturonase has been based on methods which would not distinguish between this enzyme and other pectic glycosidases. The possibility that pectic lyase or polygalacturonate lyase is involved in the invasion of red clover by Rhizobium trifolii was investigated by Macmillan and Cooke (30), who detected weak pectin lyase activity in uninoculated seedlings, but no increase in the activity was produced in inoculated seedlings. They concluded that neither of the lyases has significance in the infection process. Evidence is clearer for pectin methyl esterase activity being involved in invasion of root hairs. Subba and Sarma (50) grew seven different legumes aseptically and with homologous and heterologous rhizobia being present in the root region. Samples of root exudates were tested aseptically, and they were found to possess demonstrable pectin methyl esterase activity. The results (50), indicated that the presence of homologous rhizobia in the root region stimulated the enzyme activity of root exudates while the presence

of heterologous rhizobia often decreased the enzyme activity of the exudates. However, Subba and Sarma (50), concluded that the results obtained with Trigonella foenum-graecum did not lend themselves easily to any generalization.

Once rhizobia have infected and formed a nodule in a homologous host, the symbiosis can be effective - where the atmospheric nitrogen is fixed or ineffective if no nitrogen is fixed. There is a wide spectrum of effectiveness and ineffectiveness because of the influence of host plant species (49), and so far no single strain has proved highly effective upon all its host plants. Erdman (11) studied the efficiency of fifteen strains of Rhizobium trifolii obtained from ten Trifolium species. Each strain was inoculated into jars containing seedlings of four clover species: Trifolium alexandrum, T. fragiferum, T. subterranean and T. resupinatum. No one bacterial strain was equally effective with each of the four plant species, the variation being considerable. He concluded that maximum growth and nitrogen fixation depend upon the presence of an effective strain of Rhizobium.

Although the host factors are equally important in the success of symbiosis, there are authenticated cases where alteration of effectiveness or ineffectiveness have been achieved by factors which at first only affect the rhizobia, but can also modify rhizobia legume symbiotic association. Variation in the susceptibility of rhizobia to antibiotic substances have been reported by Schwinghamer (47) and Gupta (15) who consider that mutation for resistance to antibiotics is usually accompanied by loss of effectiveness. Schwinghamer (47) and Gupta (15) also noted that most strains are susceptible to antibiotics. In contrast, Landerkin and Lochhead (23) observed that strains of Rhizobium japonicum were less susceptible to actinomycete antibiotics than were other soil organisms tested. Hamatova (16) claimed that penicillin increased the efficiency of nearly all strains of Rhizobium

meliloti and R. trifolii and of some strains of R. lupini and R. leguminosarum. He found no relationship between the efficiency of rhizobia and their susceptibility to penicillin. Many antibiotic-resistant mutants derived from effective strains of rhizobia, mainly Rhizobium leguminosarum and Rhizobium trifolii showed some loss of effectiveness on the homologous host (48).

Holland and Parker (18) attribute the problem of clover establishment on virgin soils in Australia to be caused by antibiotic-producing fungi. In certain newly cleared soils of Western Australia, subterranean clover fails to nodulate. The plants are stunted and discolored with greatly increased anthocyanin and decreased chlorophyll in the leaves and petioles. Chemical sterilization of such a soil in the field permitted nodulation and normal growth. Water extracts from the problem soils, and the leaves of effective plants, proved toxic to Rhizobium trifolii. The problem of clover establishment on virgin soils appeared to be caused by antibiotic-producing fungi which proliferate on the organic debris remaining after the original vegetation has been removed.

Some pasture legumes, notably subterranean clover, Louisiana white clover and Kenya white clover are productive at elevations over 2438 m. in Kenya. These plants have been difficult to establish in mixtures with grasses. Kenya white clover (Trifolium semipilosum Fres.) is an herbaceous legume indigenous to the highlands of Eastern Africa and South Arabia. Attempts to introduce T. semipilosum into areas where it does not occur naturally have not been successful (32), although the future is promising. Robinson (43) attributes failure in establishment of the above legumes to competition, poor nodulation and phosphorus deficiency. Mwakha (32) points out that Kenya white clover is adapted to permanently cool tropical highlands with a mean temperature range of 10 to 13°C. throughout the year. It is important

to point out that the high altitude farming areas of Kenya consist of land at elevations of 2438 m. to 3048 m. The mean annual rainfall is over 100 mm. distributed to give a growing season of 8 to 10 months. The soils are red loams of volcanic origin with a pH range of 4.5 to 5.5. The low soil pH, which is apparently minimized, can be a sole cause of nodulation failure unless rhizobia are adapted to acidic conditions.

The sequence of steps which leads to the appearance of root nodules on leguminous plants has been studied by many workers e.g. by Nutman (40). It is only necessary to describe here the simplified sequence in lucerne: (i) development of root hairs, (ii) development of a population of rhizobia in the rhizosphere, (iii) curling and infection of root hairs, (iv) development of infection threads and (v) formation of nodules. It is well established that acidity inhibits nodulation, but the nature of the inhibition is not clearly understood, except for the recent report of Munns (37).

Inoculated red clover grown in culture solution failed to nodulate and died at pH 3.3 even if supplied with combined nitrogen, (14). Nodulation did not occur at pH 4.2 although plants continued to grow well if supplied with 1 mg. per liter nitrogen as NH_4NO_3, indicating that such an acidity prevented Rhizobium from infecting the root hair, but the low pH did not directly prevent plant growth (14). Both nodulation and growth were good at pH 5.4 to 7, which is also confirmed by Dilz (9) and Munns (37). Various Rhizobium species will nodulate at different soil pH levels e.g. alfalfa, red clover and soybean bacteria do not nodulate at pH 5.0, 4.8, 3.9 respectively.

It has been known for some time that a calcium requirement for the nodulation of legumes and that this requirement is related to the pH of the culture medium. The lower the pH, the higher the calcium requirement in the range of pH 4.6 to 5.6 (27), (29). This effect of pH and calcium concentration

occurs at the stage of nodule initiation. The effect of calcium is neither on the growth of the roots, root hair development nor are there any effects upon the rhizobia. Calcium concentrations below 0.2 mM. and pH below 4.8 inhibited nodulation at all tested levels of the other variables. In the reports (26), (27) and (29) it was also found that infections were initiated and nodulation continued normally even if the calcium concentration was raised from the inhibitory level of 0.5 mM. at pH 5.2 to the optimal concentration (8.0 mM., pH 5.2) for one day before being returned to the deficient medium. That the roots did not take up calcium to maintain the nodulation process is shown by the fact that preinoculation treatment at 8.0 mM. calcium had no effect on nodulation. Lowering the calcium concentration to 10 µM. at any stage of nodulation resulted in the cessation of the nodulation process and root growth. That Schmel, Peech and Bradfield (44) emphasized the interaction of calcium and hydrogen ions on plant growth and calcium uptake is in direct contrast to that required for nodulation. It is thus certain that the requirement for calcium is higher during the first day of infection than in its later stages. Similar findings were obtained with regard to the effect of pH on nodulation (35). At concentrations of calcium sufficiently low to inhibit nodulation completely (0.4mM. at pH 5.2) root hair curling was also completely inhibited. At the higher concentration of 0.5 mM., when nodulation was partially inhibited, root hair curling remained unaffected. It is postulated that the calcium requirement is for some activity, in the rhizosphere or on the root hair wall, that is concerned with infection. Because of the interaction between pH and the calcium requirement and the coincidence in timing of the inhibitory effects of calcium deficiency and low pH, the acid-sensitive step in the infection process appears to be connected with the calcium requirement. Similarly, there may be some connection with pectinase action (37).

Inoculation of white clover (Trifolium repens L.) with effective strains of Rhizobium and the addition of lime have been shown to increase both the vigor of the legume and the proportion of effective to inferior strains of nodule bacteria in soil. In Scotland, the presence of ineffective strains has been shown to be correlated with a low base status, and in Wales the proportion of effective strains diminished as altitude increased. The two factors that make white clover a very valuable agricultural crop are its high mineral and protein content and its contribution to the nitrogen economy of soil. It is imperative, therefore, to ensure that conditions are as near optimum as possible for both partners in the symbiosis if vigorous legume is to be established. Two of the ways of doing this are to raise the pH by liming and to provide and maintain effective strains of Rhizobium in adequate numbers. Albrecht and Davis (1) showed that under conditions of moderate calcium deficiency, the addition of lime increased the percentage of protein and the vigor of subterranean clover, the effect being similar to an addition of combined nitrogen.

In the more acid upland areas, the most important factor acting against successful nodulation is the high proportion of ineffective strains of nodule bacteria. The investigation of Jones (21) confirms this point and illustrates the further disadvantage to poor nodulation that Rhizobium is present in low numbers. Liming which generally raises the standard of fertility appears to be necessary to bring about an increase in density of the rhizobia, and initially also, the proportion of effective strains in the population.

Sometimes inoculum is imported from one country to the other for the inoculation of legumes, a practice which has encountered some nodulation failures. In certain areas, soil fertility status is such that not only do the indigenous rhizobia perform poorly, but even the imported strain may fail

to serve its purpose. Holding and King (19) state that Rhizobium strains nodulating indigenous clover plants in Scotish hill pastures were predominantly of low effectiveness. Erdman (11) provided evidence to show that in Alabama the soil contained native soybean nodule bacteria which could fix nitrogen in "unadapted" varieties of the plant. Effective strains of soybean bacteria isolated from commercial varieties in the corn belt are not generally effective on Southern soybean varieties (11). Norris (38) tested the ability of many rhizobia derived from European and African clovers to nodulate 8 African Trifolium spp. One of the 2 African clover rhizobia caused effective nodulation of the African clover whereas the European clover rhizobia, where nodules were formed, the nodules were ineffective. Norris also showed that the European clover rhizobia fail to cause nodulation in the African Trifolium spp.; thus symbiotic affinity is at a minimum. It is concluded that introduction of a new Trifolium sp. into another country is of little potential value unless there are suitable rhizobia in that country which fix nitrogen by effective symbiosis.

Since there are gradations of effectiveness and ineffectiveness, there could be a similar gradation in symbiotic specificities within the same so-called cross-inoculation group. Since effectiveness and ineffectiveness are altered by various environmental, nutritional and biochemical variables; this study aims at surveying some of these variables and how they might affect specificity within a cross-inoculation group of the Trifolium spp. It is hoped that the present series of studies will provide some insight into factors that might affect rhizobia-legume specificity.

The following factors will be surveyed and studied: (1) effect of pH on nodulation and growth pattern of axenic rhizobia cultures; (2) increase in numbers in legume rhizosphere prior to nodulation; (3) search for a non-nodulating strain within Rhizobium trifolii; (4) influence of host geographical

origin on effectiveness and (5) alteration of effectiveness as caused by rhizobia resistance to antibiotics.

MATERIALS AND METHODS

Cultures of rhizobia used

Rhizobium trifolii strain Rt_8 (wild type, effective on white clover) was obtained from the culture collection of the Department of Agronomy, Cornell University. Rhizobium trifolii strain Rt_1 (viomycin-resistant mutant) was derived from Rt_8 by A.J. Francis, Department of Agronomy, Cornell University. Rhizobium trifolii strain T_1 (wild, type, effective on red clover), T_1V_1 (viomycin-resistant mutant) and T_1N_2 (neomycin-resistant mutant) were originally supplied by Dr. E.A. Schwinghamer, Oregon State University, Corvallis, Oregon, U.S.A. Rhizobium trifolii designated as S_2, W_2, H_2 and R_2 were isolated from Trifolium fragiferum (strawberry white clover), Trifolium repens (Ladino white clover), Trifolium pratense (red clover) and Trifolium hybridum (Alsike clover) respectively. Clover plants came from Cornell University lawn, and Dr. H.A. MacDonald assisted in their identification.

All the Rhizobium species used in this study were streaked on YEM agar (see below) plates, and single colonies were selected and inoculated on YEM slants. Stock Cultures were maintained on slants of YEM medium at 30°C. and were transferred at three-month intervals.

Media

Yeast Extract Mannitol (YEM) routinely used for cultivating the rhizobia contained the following ingredients: 1.0 g. K_2HPO_4; 1.0 g. KH_2PO_4; 0.20 g. NaCl; 0.18 g. $MgSO_4$; 0.13 g. $CaSO_4.2H_2O$; 10.0 g. mannitol; 0.0003 g. $FeCl_2.4H_2O$; 1.0 g. yeast extract (Difco); 1.0 g. KNO_3; 15.0 g. agar (Difco) when required for solid medium; and 1.0 liter of deionized distilled water. The pH of the medium before autoclaving was 6.5.

Fahraeus' carbon-and-nitrogen free medium contained 0.1 g. $CaCl_2$; 0.12 g. $MgSO_4.7H_2O$; 0.1 g. KH_2PO_4; 0.15 g. $Na_2HPO_4.2H_2O$; 0.005 g. ferric citrate; traces of Mn, Cu, Zn, B, Mo, obtained by adding 2 drops of a stock

solution containing 100 mg. per ml. each of $MnCl_2$, $CuSO_4$, H_3BO_3, Na_2MoO_4; and 1.0 liter of deionized distilled water.

The YEM medium was used for growth pattern and pH studies except that K_2HPO_4 and KH_2PO_4 were each added at 0.1 g. per liter. Also, phthalate-NaOH buffer prepared according to the method of Colowick and Kaplan (5) replaced the phosphate buffer.

Nutrient broth (Difco), when used, consisted of 8 g. per liter. To the nutrient broth, 3% casamino acids were added in one study and 1.0 g. per liter of yeast extract for another study.

Plant nutrient solution prepared according to Norris (39) was made up of (a) 0.149 g. KCl; (b) 0.348 g. K_2HPO_4; (c) 0.688 g. $CaSO_4.2H_2O$; (d) 0.493 g. $MgSO_4.7H_2O$ and 1.0 liter deionized distilled water; (e) 0.157 g. $CuSO_4.5H_2O$, 0.44 g. $ZnSO_4.7H_2O$, 4.06 g. $MnSO_4.4H_2O$, 0.02 g. $(NH_4)_6Mo_7O_{24}.4H_2O$, 2.86 g. H_3BO_3 made up to 1.0 liter and added at the rate of 0.5 ml. per liter culture solution; (f) 0.5% ferrous sulfate and 0.5% citric acid mixed together and used at 0.5 ml. per liter culture solution. The solution was usually prepared in 20 liter batches by adding (a), (b) and (d) together and dissolving. Then 10 ml. of (e) and 10 ml. of (f) were added. The 13.76 g. of (c) was added and shaken up just before autoclaving for use. The nutrient solution always had a white precipitate and had to be shaken before dispensing. The pH of this solution was 7.2 immediately after preparation, it dropped to approximately 5.5 on autoclaving, and then rose slowly on standing to 5.8.

Each medium described above was sterilized by autoclaving at 121°C. at 16 lb. pressure for 20 minutes.

Antibiotics

Penicillin-G. sodium, activity 1650 units per mg., streptomycin sulfate T-3431 and neomycin sulfate T-4490 were obtained from Mann Research

Laboratory, New York, New York. Viomycin sulfate S-V-127 was a product of Parke Davis and Company, Detroit, Michigan.

The antibiotics were dissolved in water solution a few hours before use, and they were filter sterilized through millipore filters before being mixed with media presterilized by autoclave and cooled to 46°C.

Checking antibiotic resistance

YEM agar was prepared in flasks, sterilized, cooled to 45°C. in a waterbath and the antibiotic added to the agar aseptically. The agar (20 ml. per plate) was immediately poured into sterile petri dishes, cooled and allowed to solidify. Determination of the antibiotic sensitivity of wild type and mutant strains was made by streaking a loopful of cell suspension on YEM agar containing the antibiotics at a series of concentrations. It was necessary to keep the cell suspension below ca. 10^8 cells per ml., since on penicillin, in particular, higher cell concentrations made resistance appear higher. Each test included the tested bacterial strains and plates without antibiotics as controls.

The concentration of antibiotics ranged from 1 µg. per ml. to 150 µg. per ml., and the exact levels used are specified in the Results. The Rhizobium cell suspension used was a 48 hour culture grown in YEM broth. The plates were incubated at 30°C. in the dark for two days. Those organisms which grew were considered resistant to the tested concentration of antibiotic and those which did not grow were considered sensitive to the tested concentration of antibiotic.

Plants used in the study

Seeds of the following plants were obtained from Dr. J. Schiffman, Israel: Trifolium subterranean (subterranean clover), Trifolium repens (Ladino white clover) and Trifolium fragiferum (strawberry white clover).

Trifolium semipilosum L. (Kenya white clover) was obtained from Mr. D.J.A. DeSouza, Kitale, Kenya. Seeds obtained from the Department of Agronomy, Cornell University included Trifolium repens (Ladino white clover), Trifolium fragiferum (strawberry white clover), Trifolium pratense (Mammoth red clover and Pennscot red clover), Alysicarpus vaginalis (alyce clover), Melilotus alba (white sweet clover) and Medicago lupulina (black medic). All seeds were stored dry at room temperature in covered glass jars.

Surface sterilization and germination of seeds

Seeds were surface sterilized by immersion and shaking in 70% ethanol for 2 minutes followed by 1:1000 $HgCl_2$ for 5 minutes. They were then washed at least 8 times in sterile distilled water and soaked for 2 hours in the last wash. The seeds were transferred to sterile and cooled Fahraeus' carbon and nitrogen-free agar medium, allocating approximately 100 seeds per plate, except for T. subterranean, only 50 seeds were placed in each plate. The plates were incubated in a Percival (Boone, Iowa) growth chamber, kept at 25°C. on a 14 hour day length. During the 48 hour period of incubation, the plates were inverted to enable the radicle to grow away from the agar. Seedlings whose roots had attained a length of 6-8 mm. after 48 hours were considered to have good growth and were used for inoculation and nodulation tests.

Bacterial counts

Total cell counts were made by using the Petroff-Hausser counting chamber. The number of viable organisms was routinely estimated by spreading, in triplicate, 0.2 ml. of a ten-fold dilution of a bacterial suspension on to YEM agar medium. After incubation in the dark for two days at 30°C., the colonies were counted on those plates having 30 to 300 colonies. Ten-fold dilutions were carried out with sterile Fahraeus' carbon and nitrogen-free

liquid medium as the diluent.

Isolation of bacteria from nodules

A plump, healthy and firm nodule was selected. The intial step was to remove the outside contamination by disinfection and thorough washing. A nodule was carefully removed from the root leaving a small portion of the root attached to the nodule. Under running water, any soil adhering to the nodule was removed with a camel's hair brush. The nodule was put in a petri dish top containing 1:1000 $HgCl_2$ for 3 to 6 minutes, during which time it was agitated with sterile forceps.

Care was taken that the nodule was submerged most of the time in the mercuric chloride. The nodule was then removed with sterile forceps to a sterile petri dish containing 95% alcohol and then agitated for 5 minutes. It was then removed to a sterile petri dish containing sterile water where agitation was continued until the nodule was sufficiently rinsed, this last process being repeated 4 times. One ml. of sterile distilled water was added to each of 6 sterile petri dishes and the rinsed nodule transferred to petri dish number one. The nodule was crushed with sterile forceps, and the exudate was mixed with water. Five loop dilution plates from the crushed nodule exudate were prepared. These 5 plates were poured with YMI agar. The plates were rotated so as to obtain a homogenous distribution of colonies. The plates were incubated in an inverted position at 30°C. for 4 to 5 days. Several typical colonies were isolated, (as already described) and streaked on YMI agar plates. To obtain pure cultures, the streaking and incubation was repeated 4 times, or until a pure culture was isolated.

Fahraeus' technique for examination of root hair infection and nodulation

All the seedlings used were surface sterilized and germinated as already described. Then 0.2 ml. of Fahraeus' carbon and nitrogen-free medium containing 0.3 to 0.4% agar, molten at 30°C. was pipetted to one-half

of a microscope glass slide. A seedling was immediately transferred to the slide with a sterile platinum wire so that the root tip was in the agar. The cover glass (24 mm. X 40 mm.) was carefully placed over the agar and the root tip. The seed coat, if it still adhered to the cotyledons, was removed. The slide was transferred to a tube (39 mm. X 150 mm.) and covered with a 50 ml. beaker. The tube, wrapped with aluminum foil, containing 25 ml. of Fahraeus' medium, had been previously sterilized by autoclaving at 121°C. for 20 minutes, at 16 lb. pressure.

The roots were inoculated by adding a few drops of a heavy suspension (48 hour old culture) to the mineral medium. All suspensions of washed rhizobia used in the nodulation tests and subsequent plant experiments were prepared by taking a growing culture (10^8 cells per ml), centrifuging at 5000 X g. for 30 minutes and resuspending the cell pellet in sufficient sterile phosphate buffer of pH 7.0 to give the original volume. The tubes were supported with a wooden rack which also protected the roots from horizontal incident light. Incubation was at 25°C. in a Percival growth chamber, under fluorescent lamp supplying 275 foot candles and illuminated for 14 hours a day with 8 hours darkness.

After 4 days, the slides were examined for root hair deformation and infection thread formation. The slide was removed, drained of excess solution with sterile filter paper and examined under high power with the use of glycerol as an immersion fluid. The slide was then returned to the tubes, since later observation of the nodules could be accomplished by naked eye.

Previous experiments showed that there was no undesired rhizobia contamination resulting from handling the slides during microscopic examination. The plants were allowed to grow for at least 2 weeks, then, when possible, for three weeks without an external supply of nitrogen.

Test for nodulation and nitrogen fixation in petri dishes

Aseptically germinated plants were inoculated with different strains of rhizobia. Germinated seedlings were transferred to plates containing 20 ml. of Fahraeus' medium plus 0.8% agar. Three plants per plate were uniformly transferred to the surface of the agar, and the roots of the seedlings inoculated with a few drops of a water suspension of the desired organism. Uninoculated petri dishes each had three plants per plate, and they received a few drops of sterile water instead of the inoculum. The tops of the petri dishes were replaced, and a set of 30 petri dishes needed were covered with plastic to reduce evaporation. They were incubated at 25°C. in Percival growth chamber, under fluorescent lamp supplying 275 foot candles and illuminated for 14 hours a day with 8 hours of darkness.

Visual observations of inoculated and uninoculated plants were made for 1 month. Before total nitrogen in the plant material was determined, the nodule numbers were counted. The experiment was repeated twice.

Test for nodulation and nitrogen fixation using clay pots

The clay pots used had no holes at the bottom, were 12.6 cm. wide at the top, 7.7 cm. wide at the bottom and 12.6 cm. deep. They were filled with 500 to 700 g. of washed coarse sand, moistened with distilled water, covered with aluminum foil and autoclaved at 121°C. 16 lb. pressure, for 2 hours. When the pots had cooled, they were transferred to Biotronette Mark III environmental chamber (Lab-line Instruments Inc., Melrose Park, Illinois).

Surface sterilized, previously soaked clover seeds were planted, and the aluminum foil cover was used to ensure germination in the dark. After the seeds had germinated and the seedlings had emerged, the aluminum foil cover was removed. Sterile distilled water was thereafter applied as necessary to keep the sand moist but not waterlogged. At 1 to 1½ weeks of age,

plants in the pot were thinned to 20 plants per pot. At 1½ weeks of age, the plants were inoculated by adding 10 ml. of a rhizobia suspension to each pot. The inoculum for these plants was prepared by growing the bacteria in YEI broth in 500 ml. Erlenmeyer flask on a shaker at 30°C. for 2 days. Uninoculated control plants received the same amount of sterile deionized distilled water.

After 2½ weeks, all the plants received nutrient solution (39), 100 ml. being added to each pot. The growth chamber used in this study supplied both incandescent and flourescent light at an intensity of 275 foot candles. The plants were illuminated for 14 hours a day with 8 hours of darkness, with 25°C. day temperature and 23°C. night temperature.

The plants were harvested when they were 7 weeks old. They were washed free of sand and examined for the presence of nodules and general plant vigor. Each pot with 20 plants was considered as a single sample. The plants were placed on paper towels to drain moisture on roots and leaves. Each sample was chopped into small pieces using a pair of scissors then, all roots and shoot portions placed in a glass petri dish for drying at 60°C. for 1 hour. The oven was then set at 40°C. and the plants left to dry for 24 hours.

The dry plants were weighed before being ground in preparation for total nitrogen determinations.

Testing for nodulation and nitrogen fixation using Leonard jars

A Leonard jar assembly consists of a bottle (with the bottom cut out) which rests in an inverted position on a wide-mouthed bottle. In some of the assemblies, a tumbler was used. This was a slight modification from the original design described by Leonard. Modification was necessary since some of the bottle bottoms broke in the cutting process. The neck of the bottle was plugged with cotton wool and the bottle filled with washed sand.

The assembly was made from 7-Up soda bottles. When cut, the bottles were approximately 10 cm. in diameter by 26 cm. high, and the bottles held approximately 900 g. of dry sand. Inverted cut bottle tops stood in another bottle bottom 10 X 10 X 15 cm. high. The cutting of these bottles, like the plant culture tube used is in the Fahraeus' technique, was done with an electrically heated wire or diamond saw.

In preparing the Leonard jar assembly, dry sand was added to the bottle, making sure that no air lock occurred in the neck. About 100 ml. of Norris' nutrient solution was then poured into the top of the sand and allowed to run through to the jar, and remainder of the 500 ml. Norris' nutrient solution was placed in the jar. The top of the assembly (i.e. the open bottom of the inverted bottle) was capped with aluminum foil held by a rubber band. The whole assembly was wrapped with a second layer of aluminum foil and held by 3 rubber bands equidistantly placed from top to bottom.

This last modification of aluminum foil held by rubber bands prevented rocking about of the top part, it guarded against a chance contamination entering the open space between the bottle and the jar, and it also stopped excessive heating of the assembly by the sun. Each individual assembly was placed in a 10 X 10 X 30 cm. portion of a 50 X 40 cm. open box. The units were autoclaved for 2 hours at 121°C. After autoclaving, the units were transferred to the greenhouse and set up on a bench equipped with fluorescent lamps supplying light at an intensity of 275 foot candles. Artificial illumination was provided for 14 hours a day, regardless of the sunshine period, for the entire period covered by the experiments. Greenhouse temperature was 20 $\pm$ 2°C.

The techniques of handling seeds and seedlings, watering, mineral nutrition, inoculation, harvesting and preparation of plant material for dry

weight and nitrogen determinations were the same as those already described under the method where clay pots were used in growth chamber. It should be noted that Leonard jar system required watering once a week only, i.e. once watered, the plants were automatically self-irrigated. The experiment was repeated in the greenhouse under the same conditions using clover varieties from Israel i.e. Trifolium subterranean, T. repens and T. fragiferum. Only Rhizobium trifolii strain T_1 and Rt_8, which are effective on white and red clover, were used as inoculum.

Nodulation test using plant culture tubes

The plant culture tubes used were 20 cm. long and 3.5 cm. in diameter. Into each tube, 50 ml. of Fahraeus' carbon and nitrogen-free medium with 0.8% agar was poured. The top of the tubes were fitted with a Morton closure (Bellco Glass Inc., Vineland, N.J.). After autoclaving, the tubes were tilted so that the agar formed a slant as it cooled. Two-day old aseptically germinated clover seedlings were transferred aseptically to the surface of the agar. Three seedlings were transferred to each culture tube. The roots of the seedlings were inoculated with 1.0 ml. of a heavy Rhizobium cell suspension (1×10^8 cells per ml.). Ten ml. of sterile Fahraeus' carbon and nitrogen-free medium was added so that the roots were completely submerged in the medium. As soon as the Morton closures were replaced, the tubes were set up in racks which could support about 100 culture tubes. Incubation was in Biotronette Mark III environmental chamber.

After 4 days, the roots were examined visually for nodule appearance and formation. It was easy to examine the plants through the plant culture tubes and removal of the Morton closures was not necessary. The experiment was continued for 6 weeks, at the end of which time nodule numbers per plant were counted. It was necessary to replenish the nutrient solution (Fahraeus'

carbon and nitrogen-free medium) by adding 10 ml. of nutrient solution at the end of the third week. No precaution was taken to protect the roots from light, although the Morton closures cut off the incident light originating from the fluorescent tube at the top of the growth chamber.

Experimental designs, replications and treatments

In all nodulation tests and nitrogen fixation experiments, a randomized block design was employed. Allocation of random numbers was made by using a table of random numbers. In earlier experiments with clay pots in the growth chamber, it became apparent that cross contamination among cultures could occur. Consequently, in all experiments performed in the greenhouse and later ones in the growth chambers, randomization was carried out within blocks, each bacterial strain being confined to a single block.

Except as specifically stated, 3 or more replicates per treatment were the rule in all experiments in which plants were involved. When dealing with bacteria in axenic culture, at least two replicates were employed. Most experiments were repeated twice, sometimes thrice, under the same conditions to confirm the validity of the tested hypothesis.

Treatment was used to a mean clover variety inoculated with a known bacterial strain; uninoculated clover variety; clover variety to which nitrogen was applied or a particular bacterial strain cultured at some known pH, temperature etc. For each set of treatments, there was a corresponding control in which a variable was missing or substituted.

Micro-Kjeldahl method for total nitrogen

Total plant nitrogen was determined by the micro-Kjeldahl method for total nitrogen (3). The preparation of the plant material prior to analysis was performed as described in a previous section. Deionized

distilled water was used as comparison containing no nitrogen. This sample received the same treatment as the plant or any other material being analyzed. It was noticed that algal growth developed on the surface of sand employed for growing the plants, especially in those experiments performed in the greenhouse. For this reason, it was necessary to estimate what contribution, if any, the nitrogen fixed by algae would make to the nitrogen fixation. The sand used for growing the plants was analyzed for nitrogen before and after each experiment.

To determine Rhizobium cell protein, dry cell material used as sample was prepared as described in the next section. Total cell nitrogen was determined by micro-Kjeldahl method, while cell protein in mg. per ml. was calculated as follows:

Total N X 6.25 = Total protein

In order to check the accuracy of the technique of nitrogen determination, egg albumin was analyzed by the micro-Kjeldahl method. Four samples were weighed, analyzed and their protein content estimated.

Estimation of cell dry weight

Cells grown as described below were sampled for the determination of dry weight. Cultures were of the same age when they were diluted in YEM broth, (the initial growth medium), to a uniform density of 1.0. Ten ml. of cell suspension was pipetted into centrifuge tubes, and the cells were collected by centrifuging at 5000 X g. for 20 minutes. The supernatant was discarded and the filtrate washed twice and centrifuged with an equal volume of phosphate buffer, pH 7.0. The final precipitate was suspended in phosphate buffer, pH 7.0. The final volume of cell suspension was adjusted to 10.0 ml. before being poured into an aluminum weighing pan. The pans and contents were placed in an oven, dried at

85°C. for 20 hours, cooled in a desicator and reweighed. Cell dry weight per 10 ml. was computed by the difference between the first weight from the second. Dried cells obtained by this procedure were used for determination of total nitrogen.

Estimation of rhizobia numbers in the rhizosphere

The plants used included nodulated T. repens, T. fragiferum, T. semipilosum and T. pratense. The non-nodulated clovers wer Alysicarpus vaginalis, Melilotus alba and Melilotus lupulina. Rhizobium trifolii strains T_1, T_1V_1, T_1N_2, Rt_1 and Rt_8 were used as inoculum for each variety.

The experimental set up was similar to that already described under Fahraeus' techniques for examination of root hair infection and nodulation. The seedlings were inoculated so as to give an initial cell number of 1×10^5 per ml. of plant culture solution. Immediately after inoculation a 1.0 ml. sample was withdrawn and ten-fold dilutions made. Diluted cell suspension was plated on triplicate plates of YEM containing 15.0 g. agar per liter, and the plates were incubated in the dark at 30°C. for 2 days. Those plates with 30 to 300 colonies were counted; this count being given a time designation of 0. Thereafter, plate counts were made every 24 hours for the first 5 days, and also on the 7th and 9th days. On each subsequent day, the dilution was increased by a factor of 10. Direct counts of the diluted cell suspension, done in a Petroff-Hausser counting chamber, assisted in estimating the expected population. On the 9th day, all the plants were examined for nodule formation.

Growth pattern at different pH values

The cultures used for growth pattern studies, effect of growth on pH changes in culture, and the stimulation of growth by calcium were grown under identical conditions.

The medium for growing rhizobia at different pH levels has already been described. Rhizobium were grown in media with initial pH values of 4.5, 4.8, 5.0, 5.5 and 6.0. The pH of media was checked before and after autoclaving with a Beckman pH meter. Cultures were grown in duplicate 500 ml. Erlenmeyer flasks containing 200 ml. of medium and inoculated with 0.1 ml. of a washed inoculum prepared as already described. The flasks were placed on a gyrotory shaker (New Brunswick Scientific) which operated at 220 rpm. through a diameter of 3 cm.

Growth measurements were made with a Bausch and Lomb Spectronic 20 spectrophotometer. Specially matched culture tubes were used for optical density measurements. Optical density readings were taken at 660 nm. When 500 ml. Erlenmeyer flasks, equipped with matched sidearms were used, the flasks were removed at intervals from the shaker and tipped so that the liquid culture filled the sidearm. The sidearm was inserted in the spectrophotometer, the remaining parts of the flask being covered with aluminum foil to eliminate interference by external light and the optical density reading recorded. At the same time 5.0 ml. sample was pipetted from the flasks into a small test tube, and the pH of the cell suspension was measured with Beckman pH meter. When ordinary flasks were used, the sample was withdrawn into a matched test tubes and the optical density and the pH measured before discarding the sample. Uninoculated medium was used as blank.

To match the culture tubes, 18 X 150 cm. tubes were partly filled with distilled water. A "standard" tube was placed in the spectrophotometer and the pointer was set at 0.2 on the optical density scale. The remaining tubes were matched against this standard. Those tubes which differed from the standard by more than $\pm$ 0.01 optical density units were not used.

Generation time at various pH were calculated by comparing the rate of increase in growth during the logarithmic phase. Such estimation were also checked by the formula (41):

$$\text{Generation Time} = \frac{0.301}{\text{Slope}}$$

Slope refers to the slope of the line obtained by plotting the logarithm of optical density vs. time.

Stimulation of growth by calcium at low pH values

The procedure for stimulation of growth by calcium was similar to that already described above. In the previous studies, calcium was supplied to the cells in a defined medium at 0.13 g. $CaSO_4.2H_2O$ per liter. In this experiment, 2.60 g. $CaSO_4.2H_2O$ per liter was used (i.e. 20 times the normal calcium supply). There was a pH drop caused by addition of another $CaSO_4.2H_2O$, and the pH was readjusted by adding 0.2 N. NaOH until the desired pH was achieved. The pH was checked before and after autoclaving. The initial pH value of the media used were 4.5 and 5.0. Optical density and changes of pH of the media were assessed at intervals during a 3 day growing period.

Effect of excess calcium on nodulation

Clover seedlings were grown and inoculated as described under "Test for nodulation by Fahraeus' Technique." Fahraeus' carbon and nitrogen-free medium was supplemented with 1.30 g. $CaSO_4.2H_2O$ per liter as opposed to the usually applied level of 0.13 g. $CaSO_4.2H_2O$ per liter. The pH of the media was readjusted as above. As incubation continued, the seedlings were examined for appearance of the first visible nodule and the rate and degree of deformation of root hairs. After 2 weeks, the plants were examined for nodule number, size and vigor; using a comparison set of plants which received no additional calcium.

Attempt to overcome low pH effect by high amount of initial inoculum

Since the preceding studies had shown that low pH, i.e. 4.5, inhibits the growth of Rhizobium Trifolii strains T_1V_1, T_1N_2 and Rt_1, low to high initial inoculum was used to inoculate cultures grown in 500 ml. Erlenmeyer flasks containing 200 ml. of YEM broth. The size of the initial inoculum was 1×10^3, 1×10^6, 1×10^{10}, 5×10^{10} and 10×10^{10}. Reading of the optical density was taken on the 90th and 144th hour after inoculation because at an initial pH growth did not start until 72 hours after the time of inoculation.

Changes of pH in the media of inoculated clover

The initial pH of the medium in which clover and rhizobia were cultured was 4.5. The experimental methods were the same as those described under test for nodulation by Fahraeus' technique. Following inoculation, a 50 ml. sample was withdrawn from each triplicate treatment and its pH measured with the Beckman pH meter. This reading of 4.5 for all treatments was referred to as the initial pH. The sample was discarded to avoid contamination of the remaining culture. After 2 weeks, the pH of the media were measured directly from the plant culture tubes containing plants and rhizobia. This pH was referred to as the final pH. The plants were also examined for the presence of nodules. The comparison treatments consisted of media alone, media plus seedlings (without rhizobia) and media plus rhizobia (without seedlings).

Assay of indole acetic acid

Indole acetic acid was determined by the method of Tanner and Anderson (51) with some slight modifications.

Rhizobium were grown on agar slants of media containing YEM, 1% mannitol and 0.5% yeast extract. The cells were washed from the slants with a 1% sterile mannitol solution. The reaction mixture consisted of

approximately 10 X 10^6 cells per ml., 1% mannitol, 10^{-3} M DL-tryptophan, 10^{-2} M KNO_3 in a total volume of 150 ml. The container was a 250 ml. Erlenmeyer flask. The pH was adjusted to and remained between 6.5 and 7.0. The flasks were stoppered with cotton and incubated in the dark at 30°C. Aliquots were removed at 2 hour intervals and the cells removed by centrifugation at 5000 X g. for 15 minutes. The supernatant liquids were used for analytical determinations. Sterility was not maintained in the reaction mixtures. For determination of indole acetic acid, 2 drops of concentrated HCl were added to 5.0 ml. of supernatant liquid, and the solution was then extracted twice with equal volumes of absolute ether (analytical grade). The ether was evaporated, and the residue was dissolved in 3.0 ml. of distilled water. The addition of 1.5 ml. of 0.05 M. $FeCl_3$ in 35% $HClO_4$ (Salkowski Reagent), resulted in a red color characteristic of indole acetic acid. The optical density at 525 nm. and the shape of the spectrum was determined by using Bausch and Lomb spectronic 20, and Beckman DU spectrophotometers, respectively. The optical density values were converted to molar concentrations from a standard curve.

Polygalacturonase activity

The method of Munns (37) was employed to prepare the enzyme polygalacturonase from the rhizosphere of clover seedlings, and the same method was used in the assay of the enzyme's activity. The following modifications were made in the procedure of Munns (37). (a) plants were grown as described under "Test for nodulation and nitrogen fixation" using culture tubes, (b) Ostwald-Renske model 200 viscometers (Fisher Scientific Company, New York, N.Y.) with a water efflux time of between 11.2 and 11.1 seconds were used, (c) pectin (citrus) (Eastman Organic Chemicals) was employed, (d) the incubation time was 24 hours with readings being taken at intervals of 2 hours for the first 12 hours and 3 hours for the rest of the time.

The same method was used to assay for polygalacturonase activity in culture filtrates obtained from bacteria grown in pure culture.

Catalase activity

Ten ml. of actively growing cells was removed from seedling culture solution and centrifuged at 2500 X g. for 15 minutes. The precipitate was washed twice, each washing being followed by centrifugation. The washed precipitate was resuspended in 10.0 ml. of phosphate buffer, pH 7.0. The assay tubes cell suspension were mixed on the vortex junior tube mixer for 30 seconds before altering the pH. For each replicate test tubes the pH of the cell suspension was adjusted to 2.0, 3.0, 4.5, 5.0, 8.0 and 11.0. In one treatment, when catalase activity had been estimated at pH 11.0, the pH of cell suspension was lowered to 2.0 before testing for catalase. When adjusting the pH, 2N H_2SO_4 was used to lower the pH and 0.2 N. NaOH to raise it. Four drops of 3% H_2O_2 was added to the cultures in test tubes, and the evolution of O_2 was observed after 2 minutes. The results were recorded at 4 ratings: (++++) being the highest rate of O_2 evolution, (+) least evolution whole, (-) indicated no activity.

Abbreviations used

L.w	Ladino white clover
S.w	Strawberry white clover
K.w	Kenya white clover
P.r	Pennscot red clover
M.r	Mammoth red clover
Dw	Dry weight
N	Nitrogen
df	Degrees of freedom
Ms	Mean square
ss	Sum of squares
F	F test
Ns	Not significant
n	Number of observations
r	Replicates
%NU	Nitrogen expressed as percent of uninoculated treatment

EXPERIMENTAL RESULTS

A. Checking antibiotic resistance

Five Rhizobium trifolii strains T_1, T_1V_1, T_1N_2, Rt_1 and Rt_8 were checked for their antibiotic resistance. The level of resistance of mutants examined generally was about 2 to 5 times higher than that of the corresponding parent strain; increased resistance depended on the strain and on the antibiotic involved.

Table 1 shows that Rt_1, which is a mutant of Rt_8, the former strain being wild type parent and viomycin susceptible, was able to grow at 50 µg. per ml. of viomycin. Strain T_1V_1, a mutant of strain T_1 (wild type parent, which grows at 2 µg. viomycin per ml.) only grew at 10 µg. viomycin per ml. The results of Table 2, which shows comparative resistance of parent and mutant strains of rhizobia to neomycin, indicate that wild type T_1 is able to grow at 5 µg. neomycin per ml. whereas a mutant T_1N_2, derived from it grew at 20 µg. neomycin per ml. Strain T_1V_1 grew at a much higher neomycin concentration, which reflects a possibility that it might have acquired cross resistance to neomycin as a result of acquiring viomycin resistance. On the other hand, Rt_1 seemed to have acquired a cross resistance to neomycin of no more than 2 µg. neomycin per ml.

When these 5 strains were checked for their streptomycin resistance, it was found that other than Rt_1, which did not grow in the presence of even 2 µg. per ml. of streptomycin, T_1, T_1N_2 and Rt_8 were able to grow at 5 µg. streptomycin per ml. (Table 3). In this same comparison, strain T_1V_1 grew at 10 µg. streptomycin per ml.

Strain Rt_1 did not grow in penicillin as well as streptomycin. Strains Rt_8 and T_1N_2 grew in media containing 5 µg. penicillin per ml. similar to the level of streptomycin in which the strains had grown. Strain T_1V_1 grew at 10 µg. penicillin and streptomycin per ml. It is only

TABLE 1. Comparative resistance of parent and mutant strains of rhizobia to viomycin

Viomycin µg. per ml. of media	*Rhizobium trifolii* strains				
	T_1	T_1V_1	T_1N_2	Rt_1	Rt_8
0	+	+	+	+	+
2	+	+	+	+	-
5	-	+	+	+	-
10	-	+	+	+	-
20	-	-	-	+	-
50	-	-	-	+	-
100	-	-	-	-	-

\+ Growth at the indicated concentration of antibiotic.

\- No growth at the indicated concentration of antibiotic.

T_1 Parent strain of T_1V_1 (viomycin resistant and T_1N_2 (neomycin resistant) mutants.

Rt_8 Parent strain of Rt_1 (viomycin resistant) mutant.

TABLE 2. Comparative resistance of parent and mutant strains of rhizobia to neomycin

Neomycin µg. per ml. of media	*Rhizobium trifolii* strains				
	T_1	T_1V_1	T_1N_2	Rt_1	Rt_8
0	+	+	+	+	+
2	+	+	+	+	+
5	+	+	+	-	-
10	-	-	+	-	-
20	-	-	+	-	-
50	-	-	-	-	-
100	-	-	-	-	-

\+ Growth at the indicated concentration of antibiotic.

\- No growth at the indicated concentration of antibiotic.

T_1 Parent strain T_1V_1 (viomycin resistant) and T_1N_2 (neomycin resistant) mutants.

Rt_8 Parent strain of Rt_1 (viomycin resistant) mutant.

TABLE 3. Comparative resistance of parent and mutant strains of rhizobia to streptomycin

Streptomycin µg. per ml. of media	*Rhizobium trifolii* strains				
	T_1	T_1V_1	T_1N_2	Rt_1	Rt_8
0	+	+	+	+	+
2	+	+	+	-	+
5	+	+	+	-	+
10	-	+	-	-	-
20	-	-	-	-	-
50	-	-	-	-	-
100	-	-	-	-	-
150	-	-	-	-	-

\+ Growth at the indicated concentration of antibiotic.

\- No growth at the indicated concentration of antibiotic.

T_1 Parent strain of T_1V_1 (viomycin resistant) and T_1N_2 (neomycin resistant) mutants.

Rt_8 Parent strain of Rt_1 (viomycin resistant) mutant.

TABLE 4. Comparative resistance of parent and mutant strains of rhizobia to penicillin

Penicillin g. per ml. of media	*Rhizobium trifolii* strains				
	T_1	T_1V_1	T_1N_2	Rt_1	Rt_8
0	+	+	+	+	+
2	+	+	+	-	+
5	+	+	+	-	+
10	+	+	-	-	-
20	+	-	-	-	-
50	-	-	-	-	-
100	-	-	-	-	-
150	-	-	-	-	-

\+ Growth at the indicated concentration of antibiotic.

\- No growth at the indicated concentration of antibiotic.

T_1 Parent strain of T_1V_1 (viomycin resistant) and T_1N_2 (neomycin resistant) mutants.

Rt_8 Parent strain of Rt_1 (viomycin resistant) mutant.

T_1 which grew at 20 µg. penicillin per ml. as opposed to 5 µg. streptomycin per ml.

B. Colonial types of some antibiotic-resistant mutants

In testing for neomycin and streptomycin resistance, it was noticed that 2 forms of colonies appeared. The first type of colony grew in YEM agar and was observed after 24 hours. Even after 72 hours, the diameters of such colonies did not exceed 1 mm. The colonies were white, slightly raised and had an entire edge. The second type of colonies were large and attained a diameter of 7 mm. after 72 hours, but growth was not noted in YEM agar until after 48 hours. This suggests that the large mucoid colonies were more sensitive to penicillin, streptomycin, neomycin and viomycin than the small colonies. Most of the small colonies were able to grow in all antibiotic concentrations tested greater than 50 µg. per ml. of media.

The original stock cultures were streaked on YEM agar containing no antibiotic to test the appearance of the big and small colonies. Each individual strain was streaked at least 5 times, and each time the possible appearance of small and big colonies was sought. Rhizobium trifolii strains T_1, T_1V_1, Rt_1 and Rt_8 all gave rise to their individual small and big colonies respectively, when streaked and screened for small or big colonies. Strain T_1N_2 sometimes gave rise to big colonies consistently for 5 successive streaking. However, small colonies of T_1N_2 sometimes gave rise after 5 successive streaking indicating that some of the small colonies were reverting to big colonies. In the course of streaking for consistent colonies, it was also noticed that the small colonies produced very little polysaccharide and were not giving colonies typical of rhizobia.

To check whether the small colonies were rhizobia, they were tested for their ability to nodulate T. fragiferum, T. repens and T. pratense. They were all Rhizobium trifolii since they nodulated clover seedlings, when tested by the petri dish and Fahraeus' tube method. The big colonies nodulated the same clover seedlings too. The small colonies have been described by D.H. Hubbel, A.J. Francis (personal communication) and by Macgregor (31). Those of Hubbel did not form nodules. Morphology of the large and small colonies appeared to be the same. Microscopic examination of the cells showed that they were all gram negative, non-spore forming rods, 0.5 to 0.9 . wide and 1.2 to 3.0 . long.

C. Testing for nodulation and nitrogen fixation in various media in growth chamber and greenhouse

One of the techniques used in testing for nodulation as well as nitrogen fixation was the petri dish method. The results reported in Table 5 show the dry weight and nitrogen when 4 clover varieties were inoculated with Rhizobium trifolii strain S_2, W_2, H_2 and R_2. These rhizobia were isolated from clover nodules obtained from Cornell University clover and grass lawn. Excellent nodulation was obtained by these strains on all the clover varieties on which they were tested.

In the petri dish method, as employed here, the appearance of the first nodules was observed on the 8th day. After 2 weeks, many large nodules had been formed. Typical effective clover nodules were oblong, about 1 cm. long, 0.3 cm. in diameter and pink or red in color. Effective nodules were generally distributed along the main tap root, with very few on the lateral roots. The ineffective nodules were half the size of the effective ones, whitish to brown in color, and tend to be distributed generally on the tap and lateral roots.

Plants grown in petri dishes showed signs of effectiveness

TABLE 5. Dry weight and nitrogen content of 4 clover varieties inoculated with 4 rhizobia strains and grown in petri dishes

Rhizobium trifolii strain	T. fragiferum (S.w)		T. repens (L.w)		T. pratense (P.r)		T. pratense (K.r)		Treatment mean	
	Dw	N	Dw	N	Dw	N	Dw	N	Dw	N
Uninoculated	2.50	0.09	2.15	0.04	2.98	0.07	3.10	0.08	2.68	0.07
S_2	1.80	0.08	2.08	0.05	3.03	0.12	2.55	0.04	2.38	0.07
W_2	2.22	0.08	1.91	0.04	2.42	0.19	2.90	0.11	2.36	0.08
H_2	2.33	0.08	1.83	0.03	2.75	0.10	2.25	0.08	2.29	0.07
R_2	2.98	0.08	1.06	0.03	2.46	0.09	2.60	0.05	2.28	0.06
Variety mean	2.38	0.08	1.81	0.04	2.74	0.09	2.68	0.07		

Analysis of variance

Source	df	Dw Ms	Dw F	N Ms	N F
Mean	1	342.67		0.008	8.00**
Variety	3	3.46	13.80**	0.001	1.00 NS
Strains	4	0.20	0.80 NS	0.001	1.00 NS
Variety X strains	12	0.49	1.96 NS		
Error	40	0.25		0.001	

** $P<0.01$

Duncan's new multiple range test

	Ladino white (L.w)	Strawberry white (S.w)	Mammoth red (M.r)	Pennscot red (P.r)
Dry weight	1.81	2.38	2.68	2.74
Nitrogen	0.04	0.07	0.08	0.09

Dw* Dry weight in mg. per plant

N* Nitrogen in mg. per plant

Figures underlined are different from each (Duncan's test)

or ineffectiveness after about 1 month. Where an ineffective symbiotic association occurred, the plants were yellow, weak and had smaller leaves, weak stems unlike effectively nodulated, green vigorous plants.

In testing for nodulation and nitrogen fixation in petri dishes, it was noticed that Trifolium semipilosum (Kenya white clover), inoculated with the various rhizobia strains died after about 3 weeks. The seedlings grew normally for the first 2 weeks, then weakening in plant vigor, followed by yellowing, browning and death during the the 3rd week. In some treatments, 1 plant out of 3 per petri dish survived.

Although all the clover varieties inoculated with Rhizobium trifolii strains S_2, W_2, H_2 and R_2 formed effective associations, the degree of effectiveness varied, as can be seen from the levels of nitrogen fixed (Table 5). Visual observation confirmed that, in some treatments, the plants were pale green tending to yellow, indicating partial effectiveness. The efficiency of petri dishes for testing nodulation will be evaluated under the discussion section, but it suffice to mention that plants did not grow to big enough size due to physical limitation of space.

Rhizobium trifolii strains T_1 and Rt_8 originally supplied by (Dr. Schwinghamer), were known to be effective on red and white clover, respectively. It was however, not specified by Dr. Schwinghamer as to which varieties of clovers the strains were effective on. Table 6 shows the nitrogen content in mg. per plant of 5 clover varieties inoculated with 5 rhizobia strains. The plants were grown in clay pots in growth chamber. From the table, it is clear Rhizobium trifolii strains T_1 and Rt_8 were effective on Trifolium fragiferum (strawberry white clover), T. repens (Ladino white clover) and T. pratense (Pennscot and Mammoth red clovers).

This not only confirmed the already known information that strains T_1 and Rt_8 were effective on red and white clovers respectively, but also

TABLE 6. Nitrogen content of five clover varieties inoculated with five rhizobia strains

Rhizobium trifolii strains	Clover varieties										Strain mean	
	T. semipilosum (K.w)		T. fragiferum (S.w)		T. repens (L.w)		T. pratense (P.r)		T. pratense (M.r)			
	N	%NU	N	%NU	N	%NU	N	%NU	N	%NU	N	%NU
uninoculated	0.09	100	0.16	100	0.15	100	0.36	100	0.49	100	0.25	100
T_1V_1	0.06	66	0.19	119	0.15	100	0.23	64	0.28	57	0.81	72
T_1N_2	0.07	77	0.19	119	0.16	107	0.39	103	0.55	112	0.27	103
Rt_1	0.07	77	0.15	94	0.18	120	0.42	117	0.39	80	0.23	92
Rt_8	0.06	66	0.72	450	0.99	660	1.15	319	1.58	322	0.89	356
T_1	0.09	100	1.02	637	2.16	440	1.82	505	1.72	351	1.36	544
	0.06		0.42		0.67		0.73		0.83			

Analysis of variance

Source	df	Ms	F. Test
Mean	1	17.02	
variety	4	2.92	16.23 **
strains	5	0.89	4.90 **
variety X strains	20	0.21	1.20
error	19	0.18	

n = 59, r = 2

** $P<0.01$

Varieties: Duncan's multiple range test

T. semipilosum (K.w)	T. fragiferum (S.w)	T. repens (L.w)	T. pratense (P.r)	T. pratense (M.r)
0.06	0.42	0.67	0.73	0.83

%NU = Nitrogen expressed as percentage of uninoculated treatment.
n = 59, r = 2, ** P 0.01
The plants were grown in clay pots and in rowth chambor.
N* = Nitrogen in mg. per plant.

that the reverse case hold true for the clovers already mentioned and tested. On the other hand the 2 strains, T_1 and Rt_8, which were confirmed to be effective on white clovers, proved to be ineffective on Trifolium semipilosum (Kenya white clover).

It was also known from earlier work that Rhizobium trifolii strains T_1, T_1N_2 and Rt_1 were antibiotic-resistant mutants which had lost their effectiveness on red and white clovers as a result of acquiring antibiotic resistance. The results of Table 6 clearly indicate that these strains were ineffective on all the 5 clover varieties tested. At 30 days from the time of planting, it was visually possible to detect the effective from ineffective associations by plant vigor and color. By the end of 50 days, when the plants were harvested, the ineffectively nodulated plants were yellow, stunted and weak, with many small white and green nodules. The effectively nodulated plants were deep green, vigorous, healthy and had few big pinkish to red nodules.

The data in Table 7 reveal more or less similar results as the previous table, although here, the plants were grown in Leonard jars in greenhouse. In this table, the dry weight and nitrogen are reported. Since dry weight and nitrogen contents of plants have a high positive correlation, it is appropriate to report either nitrogen or dry weight as a measure of effectiveness or ineffectiveness. The correlation between nitrogen and dry weight of plants was calculated from Tables 7 and 8.

Table 7, like Table 6, shows that Rhizobium trifolii strain T_1 is superior to Rhizobium trifolii strain Rt_8 in nitrogen fixation ability, although they are both effective on the tested varieties of red and white clovers, except for the Kenya white clover. In examining individual varietal differences, T. pratense (Mammoth and Pennscot red clovers)

TABLE 7. Dry weight and nitrogen content of five clover varieties inoculated with five rhizobia strains, grown in Leonard jars

Rhizobium trifolii strains	Clover varieties										strain mean	
	T. semipilosum (Kenya white		T. fragiferum (strawberry white)		T. repens (Ladino white)		T. pratense (Pennscot red)		T. pratense (Mammoth red)			
	Dw	N	Dw	N	Dw	N	Dw	N	Dw	N	Dw	N
Rt_1	6.60	0.09	5.20	0.08	5.40	0.08	5.45	0.15	15.50	0.18	7.73	0.11
T_1N_2	5.75	0.10	5.60	0.08	5.95	0.08	12.20	0.15	30.90	0.21	9.71	0.11
T_1V_1	5.05	0.08	5.00	0.07	7.10	0.09	13.10	0.22	18.65	0.28	9.78	0.15
Rt_8	6.35	0.12	18.10	0.58	49.35	1.43	48.60	1.28	61.75	0.81	38.90	1.10
T_1	7.00	0.13	28.70	0.93	35.00	1.24	56.75	1.60	77.90	2.12	44.86	1.32
Variety mean	5.92	0.10	11.90	0.32	20.56	0.65	29.11	0.72	42.07	0.99		

Analysis of variance

Source	df	Dw Ms	Dw F	N Ms	N F
Mean	1	22031.41		13.860	
Varieties	4	1836.91	36.97**	1.112	18.23**
Strains	4	3003.03	60.43**	3.297	54.05**
Variety x strain	16	141.27	2.84*	0.139	2.27*
Error	21	49.69		0.061	

** $P < 0.01$
* $P < 0.05$

Duncan's new multiple range test

	Rt_1	T_1N_2	T_1V_1	Rt_8	T_1
Dw	7.73	9.71	9.78	38.90	44.86
N	0.11	0.11	0.15	1.10	1.32

Dw = Dry weight in mg. per plant.
N = Nitrogen in mg. per plant.
Figures underlined together are significanly different according to Duncan's test.

attained much higher dry weight and fixed more nitrogen per plant by virtue of the fact that they are bigger clovers. Similarly T. fragiferum (strawberry white clover) and T. repens (Ladino white clover) fixed less nitrogen in comparison to the red clovers, because the former are much smaller varieties.

Test for nodulation and nitrogen fixation of clover species obtained from Israel

In the previous results, it had been demonstrated that Rhizobium trifolii strains T_1 and Rt_8 were ineffective on white clover obtained from Kenya. The hypothesis that rhizobia adapted to nodulate temperate clover species may not necessarily be effective on similar species from different climatic zones was tested. The results of Table 8 are dry weight and nitrogen contents in mg. per plant obtained when T. subterranean (subterranean clover), T. repens (Ladino white clover) and T. fragiferum (strawberry white clover), all from Israel, were inoculated with Rhizobium trifolii strains T_1 and Rt_8. Although the dry weight and nitrogen value for subterranean clover seemed to be high, this clover is much bigger clover than any of the others tested. Otherwise the dry weight and nitrogen content are comparable to those obtained when the ineffective rhizobia, i.e. strains T_1V_1, T_1N_2 etc., were used to inoculate white and red clovers. When nitrogen contents of plants inoculated with strains T_1 and Rt_8 were expressed as percentage of nitrogen content of uninoculated plants, it became clear that no more than 8% increase in nitrogen content was obtained with varieties on which strains T_1 and Rt_8 were ineffective. Where strains T_1 and Rt_8 were effective, an increase to the order of 500 percent was obtained.

Ineffectiveness of these strains T_1 and Rt_8 on the Israel clover

TABLE 8. Dry weight and nitrogen content of 3 clover varieties inoculated with R. trifolii strains T_1 and Rt_8

Rhizobium trifolii strain	Clover varieties: T. subterranean		T. fragiferum		T. repens		Strain mean		%NU
	Dw	N	Dw	N	Dw	N	Dw	N	
Uninoculated	58.62	1.14	6.21	0.10	4.61	0.07	23.15	0.44	100
Rt_8	58.33	1.26	9.39	0.19	4.69	0.10	24.14	0.51	104
T_1	61.00	1.25	9.23	0.18	7.21	0.13	25.81	0.52	108
	59.31	1.23	8.28	0.16	5.50	0.10	75.97	6.15	

Analysis of variance

Source	df	Dry weight: ss	Ms	F test		Nitrogen: Ms	F test
Block (varieties)	2	22036.31	11018.15	342.780	P<0.001	4.240	114.59*
Treatment (strain)	2	43.60	21.80	6.77	P<0.005	0.025	6.75**
Experimental error	4	16.70	4.18	1.30	P<0.05	0.0025	0.67NS
Sampling error	27	87.04	3.22			0.0037	

Duncan's new multiple range test

Uninoculated	Rt_8	T_1	No significant differences
23.15	24.14	25.81	

Dw = Dry weight in mg. per plant.
N = Nitrogen in mg. per plant.
%NU = Nitrogen expressed as percentage of uninoculated plants.
Plants were grown in Leonard jars in greenhouse.

varieties was also apparent in the greenhouse 1 month after planting because the inoculated plants, like the uninoculated ones, looked yellow and stunted. Nodulation of the plants grown in Leonard jars as well as those in plant culture tubes was good (Table 12). In the treatment to which NH_4NO_3 was applied, 800 mg. nitrogen per pot, the plants were deep green, vigorous and healthy, showing that the plants could grow in the absence of rhizobia, provided nitrogen was available. The dry weight and nitrogen content of plants supplied with external mineral nitrogen are reported in Table 9. These results were part of the same experiment reported in Table 8, but they were not included in the analysis of variance of Table 8 since they would introduce a large cummulative error and consequently inflate the analysis of variance.

Table 10 shows percent nitrogen in 5 clover varieties inoculated with 5 rhizobia strains and grown in Leonard jar in the greenhouse. Strawberry and Ladino white clovers inoculated with strain T_1 fixed more nitrogen per plant than Pennscot and Mammoth red clovers. Similarly, Ladino and strawberry white clovers inoculated with strain Rt_8 fixed more nitrogen per plant than Pennscot and Mammoth red clovers. The table also shows that strains T_1 and Rt_8 were ineffective on Kenya white clover.

Table 11 shows percent of nitrogen in clover varieties from different world climatic regions and inoculated with effective rhizobia strains T_1 and Rt_8. The results show that strains T_1 and Rt_8 were effective on Ladino, strawberry, Pennscot and Mammoth red clovers, all of which had been grown in New York State for a long time. Rhizobium trifolii strains T_1 and Rt_8 were ineffective on strawberry, Ladino white and subterranean clovers, whose seeds came from Israel. Kenya white clover, whose seeds came from Kenya was ineffectively nodulated by strains T_1 and Rt_8.

Table 9. Dry weight and nitrogen of 3 uninoculated clover varieties grown in Leonard jars in greenhouse with nitrogen applied at 800 mg. per pot

	Clover varieties					
	T. subterraneam		T. fragiferum		T. repens	
Reps	Dw	N	Dw	N	Dw	N
1	192.13	15.60	20.64	1.68	17.40	1.22
2	189.63	15.13	19.00	1.36	18.36	1.42
3	186.50	15.92	19.68	1.54	19.80	1.53
4	191.50	15.54	19.47	1.48	17.56	1.35
Mean	189.94	15.55	19.70	1.51	18.28	1.38

Dw* Dry weight in mg. per plant.

N* Nitrogen in mg. per plant.

TABLE 10. Percent of nitrogen in 5 clover varieties inoculated with 5 rhizobia strains and grown in Leonard jars in the greenhouse

Clover varieties	*Rhizobium trifolii* strains					Variety mean
	T_1	T_1V_1	T_1N_2	Rt_1	Rt_8	
T. fragiferum (strawberry white)	3.24	1.40	1.43	1.53	3.20	2.68
T. repens (Ladino white)	3.54	1.26	1.34	1.48	2.90	3.16
T. pratense (Pennscot red)	2.81	1.67	1.22	1.23	2.63	2.47
T. pratense (Mammoth red)	2.72	1.50	0.70	1.15	2.93	2.35
T. semipilosum (Kenya white)	1.86	1.58	1.73	1.50	1.89	1.69
Strain mean	2.94	1.53	1.13	1.42	2.82	

Data computed from Table 7.

TABLE 11. Percent of nitrogen in clover varieties from different world climatic regions and inoculated with effective rhizobia

Clover varieties	Nodulation	Origin of varieties	Climatic area	Rhizobium trifolii strains T_1	Rhizobium trifolii strains Rt_8	Uninoculated
T. fragiferum (strawberry white)	Effective	New York State	Temperate	3.24	3.20	1.08
T. repens (Ladino white)	Effective	New York State	Temperate	3.54	2.90	1.02
T. pratense (Pennscot red)	Effective	New York State	Temperate	2.81	2.63	1.06
T. pratense (Mammoth red)	Effective	New York State	Temperate	2.72	2.93	1.03
T. fragiferum (strawberry white)	Ineffective	Israel	Sub-tropical	1.95	2.02	1.61
T. repens (Ladino white)	Ineffective	Israel	Sub-tropical	1.80	2.13	1.52
T. subterraneum (subterranean)	Ineffective	Israel	Sub-tropical	2.04	2.16	1.94
T. semipilosum (Kenya white)	Ineffective	Kenya	Tropical	1.86	1.89	1.53

Nodule number as an index of effective and ineffectiveness

It is well-known that for every genus of nodulated legume, the number of nodules per plant is an indication of ineffectiveness. By this criteria, as a rule, ineffectively nodulated plants have more nodules per plant than the effectively nodulated ones. Table 12 shows the number of nodules per plant of effectively and ineffectively nodulated plants. These were nodule counts made on plants grown in both greenhouse and growth chamber. The plants were also rated effectively or ineffectively nodulated by their yellowness, vigor, dry weight and nitrogen analysis.

It was noted that Kenya white clover grown in plant culture tubes by the Fahraeus' technique and also in petri dishes formed very few nodules. If the effectiveness of the plants were rated by the criteria of nodule number, erroneous conclusions would be drawn. However, Kenya white clover grown in clay pots and Leonard jars, in both growth chamber and greenhouse, formed many nodules. Table 13 shows the number of nodules per plant of 4 clover varieties inoculated with 5 R. trifolii strains, all of which were ineffective on the tested clovers. In the Kenya white clover and strawberry white clover, the number of nodules per plant would lead one to a wrong conclusion, namely that these are effectively nodulated plants. On the other hand, Ladino white clover and subterranean clover formed many nodules which were indicative of ineffectively nodulated plants.

Nodule number and shoot to root ratio as an index of effectiveness

The correlation of nodule number and ineffective or effectiveness has already been mentioned. An attempt was made to assess the correlation of shoot to root ratio as an index of effectiveness and ineffectiveness (Table 14). The results reported for shoot to root ratio are total shoot length of individual plants divided by total root length of the same plant. The ineffectively nodulated plants had remarkably long roots. In the case

TABLE 12. Number of nodules in clover varieties inoculated in petri dishes with effective and ineffective Rhizobium trifolii strains

Clover varieties	Strains of Rhizobium trifolii					Uninoculated control
	T_1	T_1V_1	T_1N_2	Rt_1	Rt_8	
T. fragiferum (Strawberry white)	3	17	17	20	4	0
T. repens (Ladino white)	4	21	16	18	6	0
T. semipilosum (Kenya white)	13	11	19	23	14	0
T. pratense (Pennscot red)	3	14	18	20	4	0
T. pratense (Mammoth red)	5	18	17	20	6	0

Nodule numbers were counted at three weeks after inoculation.

Results are the mean of three replicates.

TABLE 13. Nodule numbers per plant of three clover varieties grown in plant culture tubes in the growth chamber

Clover varieties	*Rhizobium trifolii* strains				
	T_1	T_1V_1	T_1N_2	Rt_1	Rt_3
T. semipilosum (Kenya white clover)	3	3	6	4	8
T. fragiferum (strawberry white clover)	5	4	3	2	9
T. repens (Ladino white clover)	4	8	14	17	38
T. subterranean (subterranean clover)	26	39	33	41	36

Nodules were counted fifty days after the time of inoculation.

Results are the mean of three replicates.

TABLE 14. Nodule number and shoot root ratio per plant of effectively and ineffectively nodulated clover plants

Clover varieties	Rhizobium trifolii strains									
	T_1		T_1V_1		T_1N_2		Rt_1		Rt_3	
	Nodule No.	shoot root ratio	Nodule No.	shoot root ratio	Nodule No.	shoot root ratio	Nodule No.	shoot root ratio	Nodule No.	shoot root ratio
T. fragiferum (strawberry white)	3	1.2	27	0.29	25	0.25	26	0.20	3	1.0
T. repens (Ladino white)	4	1.2	35	0.25	22	0.33	22	0.16	4	1.0
T. pratense (Pennscot red)	3	1.0	32	0.25	21	0.29	25	0.29	7	1.50
T. pratense (Mammoth red)	4	1.6	29	0.43	25	0.50	30	0.42	6	2.0
T. semivillosum (Kenya white)	17	0.33	18	0.40	18	0.40	20	0.17	18	0.17
Uninoculated	0	0.27	0	0.32	0	0.36	0	0.21	0	0.19

Numbers are mean of three replicates. Plants were grown in Leonard jars in greenhouse.

The shoot to root ratios are the total lengths of shoots divided by the total lengths of roots in every replicate.

of plants grown in Leonard jars, the roots of ineffectively nodulated plants were 3 to 4 times as long as those of effectively nodulated ones. There was not much difference between the shoot length of effectively and ineffectively nodulated plants. The high shoot to root ratio of effectively nodulated plants is therefore due to the fact that they had longer shoots than roots, whereas in the ineffectively nodulated plants, the roots were about four-fold as long as the shoot lengths. Uninoculated plants also exhibited low shoot to root ratios, as did the ineffectively nodulated plants.

Increase of rhizobia numbers in the legume root rhizosphere

One of the preceding steps in root hair infection is the increase in rhizobium numbers in the rhizosphere of homologous and heterologous legume host. The step has been characterized as being non-specific. Tables 15 and 16 show the increase in numbers of 5 Rhizobium trifolii strains in the rhizosphere of homologous and 3 heterologous clover varieties. It is clear from the tables that the rhizobia numbers increase from an initial count of 1×10^5 cells per ml. at the time of inoculation to about 1×10^6 cells per ml. on the 2nd day. The increase in cell number is extremely rapid between the 1st and 3rd day, and by the 4th day a maximum cell count of 1×10^8 cells per ml. had been attained. The cell numbers remain at this concentration of 1×10^8 cells per ml. until the 9th day, when the last cell count was made. It has been reported by other workers that once the maximum cell number has been reached, the number remains at that level for 1 month (40), (10).

Formation of tumor-like structures by Rhizobium trifolii mutants on Alysicarpus vaginalis (Alyce clover), has been reported by Macgregor (31). In this study, no tumor-like structures were observed on any of the non-nodulated clovers. However, it was observed that root branching was more prolific in the non-nodulated plants than in the nodulated ones.

TABLE 15. Increase in numbers of Rhizobium trifolii strains in rhizosphere of 5 nodulated clover varieties

Clover varieties	R. trifolii strains	Days after inoculation						
		1	2	3	4	5	7	9
T. fragiferum (strawberry white)	T_1	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8	1×10^7
	T_1V_1	1×10^6	1×10^6	1×10^7	2×10^8	2×10^7	2×10^8	2×10^8
	T_1N_2	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8	1×10^8
	Rt_1	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8	1×10^8
	Rt_8	1×10^6	1×10^7	1×10^8	1×10	1×10^8	1×10^8	1×10^8
T. repens (Ladino white)	T_1	1×10^6	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^7
	T_1V_1	1×10^6	1×10^7	1×10^8	1×10^8	1×10^9	1×10^7	1×10^8
	T_1N_2	1×10^6	1×10^7	1×10^8	1×10^8	2×10^7	1×10^8	1×10^8
	Rt_1	1×10^6	1×10^7	1×10^8	1×10^8	1×10^9	1×10^8	1×10^8
	Rt_8	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8	1×10^8
T. pratense (Tennscot red)	T_1	1×10^6	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8
	T_1V_1	1×10^6	1.5×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8
	T_1N_2	1×10^6	$.8 \times 10^6$	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8
	Rt_1	1×10^6	1×10^7	1×10^8	1×10^8	1×10^9	1×10^9	1×10^8
	Rt_8	1×10^6	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8
T. pratense (Mammoth red)	T_1	1×10^6	1.5×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8
	T_1V_1	1×10^6	1×10^7	1×10^7	1×10^8	1×10^8	1.5×10^8	2×10^8
	T_1N_2	1×10^6	1×10^6	1×10^7	1×10^7	1×10^8	1×10^8	1×10^8
	Rt_1	1×10^6	1×10^7	1.5×10^7	2×10^7	1×10^8	1×10^8	1×10^8
	Rt_8	1×10^5	$.8 \times 10^6$	1×10^7	1×10^7	5×10^7	2×10^7	1×10^8

Counts refer to cell density per ml.

TABLE 16. Increase in numbers of Rhizobium trifolii strains in rhizosphere of one nodulated and three non-nodulated clover varieties

Clover varieties	R. trifolii strains	Days after inoculation						
		1	2	3	4	5	7	9
T. semipilosum (Kenya white)	T_1	1×10^6	1×10^7	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8
	T_1V_1	$.8 \times 10^6$	1×10^6	1×10^7	1×10^8	1×10^8	1.5×10^8	1×10^8
	T_1N_2	$.7 \times 10^6$	1×10^6	1×10^7	1×10^7	1×10^8	2×10^8	2×10^8
	Rt_1	$.8 \times 10^6$	1×10^6	1.5×10^6	1×10^7	1×10^8	1×10^8	1×10^8
	Rt_8	$.5 \times 10^6$	1×10^6	1.5×10^6	2×10^6	1×10^7	1×10^7	1×10^8
Alysicarpus vaginalis (Alyce clover)	T_1	$.8 \times 10^6$	1×10^6	1.5×10^6	2×10^6	5×10^6	1×10^7	1×10^7
	T_1V_1	$.8 \times 10^6$	1×10^6	1×10^7	1×10^8	1×10^7	1×10^8	1×10^8
	T_1N_2	$.5 \times 10^6$	1×10^6	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8
	Rt_1	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8	1×10^8
	Rt_8	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^7	1×10^8
Melilotus alba (sweet white clover)	T_1	$.5 \times 10^6$	1×10^6	1×10^7	1×10^7	1.5×10^7	1×10^8	1×10^8
	T_1V_1	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8	1×10^8
	T_1N_2	1×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8	1×10^8
	Rt_1	1×10^6	2×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8
	Rt_8	$.5 \times 10^6$	1×10^6	2×10^6	1×10^7	1.5×10^7	1×10^8	1×10^8
Medicago lupulina	T_1	1×10^6	1×10^7	1×10^7	2×10^7	5×10^7	1×10^8	1×10^8
	T_1V_1	1×10^6	2×10^6	1×10^7	1×10^8	1×10^8	1×10^8	1×10^8
	T_1N_2	$.6 \times 10^6$	1×10^7	1.5×10^7	2×10^7	1×10^8	1×10^8	1×10^7
	Rt_1	1×10^6	1×10^7	1.5×10^7	1×10^8	1×10^8	1×10^8	1×10^8
	Rt_8	$.4 \times 10^6$	1×10^7	1×10^7	1×10^8	1×10^7	1×10^8	1×10^8

Counts refer to cell density per ml.

Prolific branching of roots was also observed in uninoculated, but normally nodulated clover varieties.

Tryptophan is converted to indole pyruvic acid by oxidative deamination or transamination. The indole pyruvic acid then undergoes oxidative decarboxylation to indole acetaldehyde; which on oxidation yields indole acetic acid. Both in plants and animals, the oxidative decarboxylation of indole pyruvic acid is the major route of indole acetic acid formation. Rhizobia do not have tryptophan decarboxylase, so that they cannot carry out decarboxylation of tryptophan to form tryptamine. Implication of indole acetic acid as a specific factor in root hair infection has been ruled out (10), (40), but its presence and activity in the legume rhizosphere should not be ignored. Since rhizobia oxidize tryptophan in axenic culture extremely fast, as shown by the data given in Figure 1. It is possible that formation of indole acetic acid may be appreciable in the root rhizosphere, especially if a legume plant excretes substantial quantities of tryptophan.

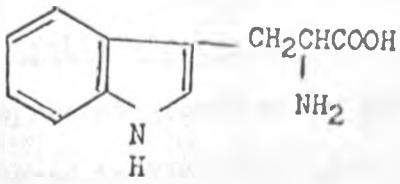
Nodulation of clover varieties at different pH values

Before testing for nodulation at different pH values, it was necessary to check the pH changes in the plant culture solution (Fahraeus' carbon and nitrogen-free medium). The results of Table 17 show that seedlings inoculated with different strains of Rhizobium have the pH of their rooting media raised by about 0.4 of a pH unit. Even the uninoculated seedlings were able to raise the pH of the media to a similar extent as the inoculated ones. Rhizobium trifolii inoculated into Fahraeus' carbon and nitrogen-free medium did not change the pH, probably because the bacteria could not grow as there was no carbon, energy and nitrogen source.

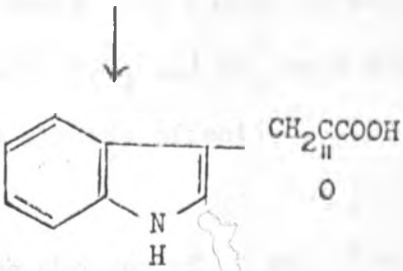
In testing for nodulation at different pH levels, it was therefore necessary to add a few drops of 0.2N H_2SO_4 every day for the first 14 days

Production of indole acetic acid from tryptophan by rhizobia

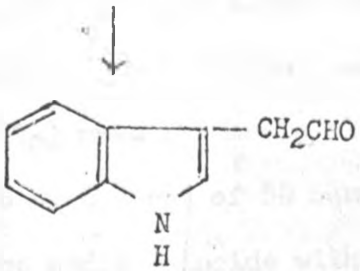
All rhizobia are able to produce indole acetic acid (IAA) from tryptophan since they possess the enzyme tryptophanase.



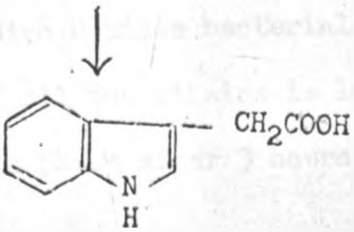
Tryptophan



Indole pyruvic acid



Indole acetaldehyde



Indole acetic acid

to keep the pH of solution at the desired level. The data of Table 18 show that at pH 4.5 there was no nodulation at all. At pH 5.0, however, some seedlings were nodulated, and at pH 6.0 all the tested seedlings were nodulated.

It should be pointed out that the seedlings grew perfectly well at all the pH levels employed, indicating that low pH affects only the rhizobia and not the seedlings.

Growth of Rhizobium trifolii strains at various pH levels

The results represent growth of Rhizobium trifolii strains T_1, T_1V_1, T_1N_2, Rt_1 and Rt_8 cultured in YEM broth with phthalate buffer. The initial pH was set at the desired level and allowed to change as growth of the bacteria progressed. Figure 2 shows growth of the strains in media with an initial pH of 6.0. There is not much difference in growth rates, although the ineffective mutants T_1V_1 and Rt_1 were faster growing than one ineffective mutant T_1N_2 and all the effective, wild type, parent strains T_1 and Rt_8.

Corresponding changes of pH measured during bacterial growth indicate that the 2 fast-growing antibiotic-resistant, ineffective mutants, T_1V_1 and Rt_1 first make the media acidic, then, in the case of Rt_1, alkaline. However, T_1V_1 lowers the pH to 5.8, and the medium remains acidic for the entire period of 80 hours. As Figure 3 shows, the slow growing strains (i.e. T_1N_2, T_1 and Rt_8) make the media alkaline, and this trend continues to a pH of about 7.8 by the end of 80 hours. It is also noteworthy that the pH changes of the media coincide with the logarithmic phase of all the strains.

Figure 4, which depicts bacterial growth at pH 5.5, shows that the logarithmic phase of all the strains is longer. At pH 6.0, T_1V_1 and Rt_1 began the logarithmic phase after 3 hours, whereas at pH 5.5, onset of

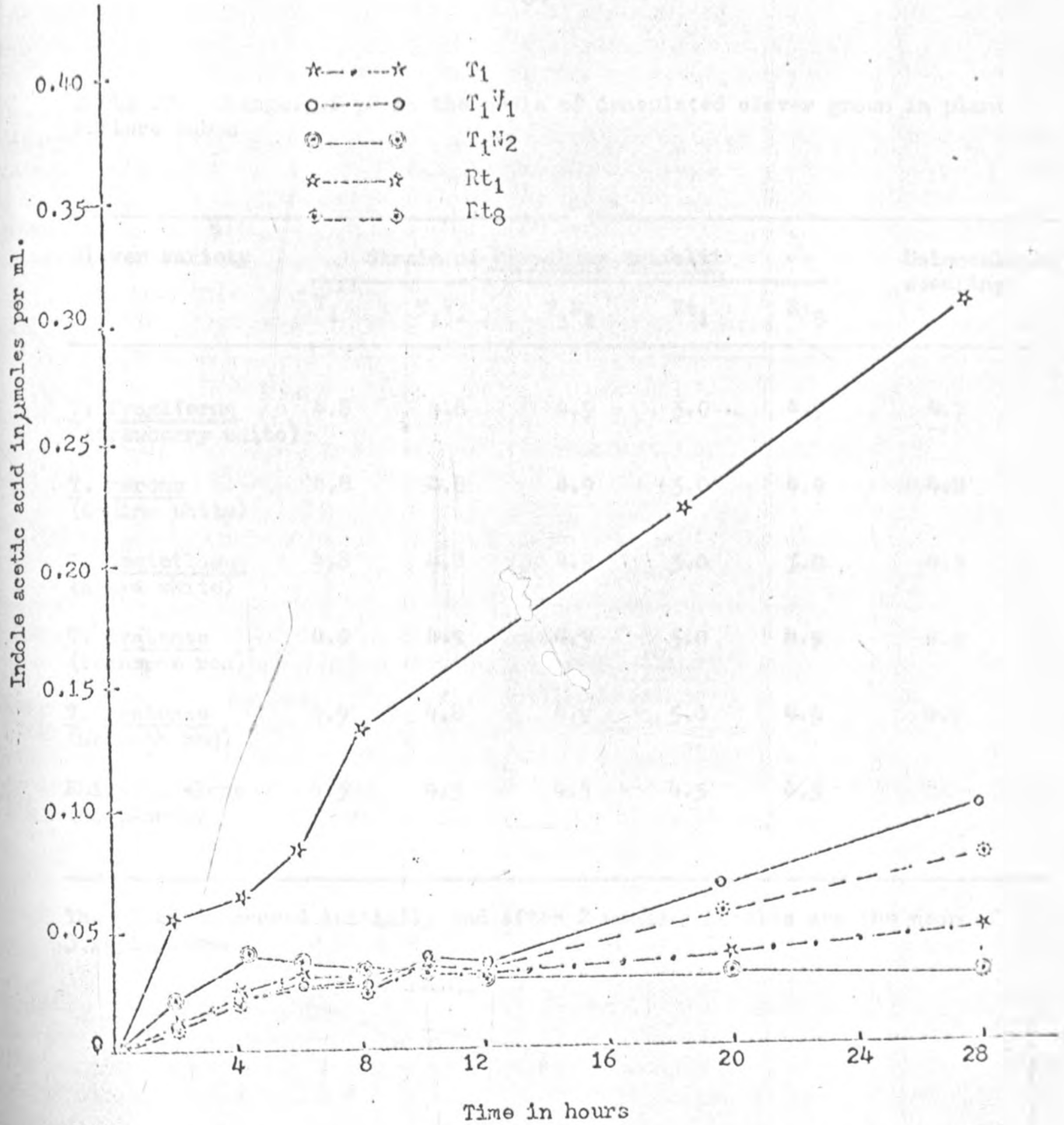


FIG. 1. Production of indole acetic acid from tryptophan by five strains of Rhizobium trifolii.

TABLE 17. Changes of pH in the media of inoculated clover grown in plant culture tubes

Clover variety	Strain of *Rhizobium trifolii*					Uninoculated seedlings
	T_1	T_1V_1	T_1N_2	Rt_1	Rt_8	
T. fragiferum (strawberry white)	4.8	4.8	4.9	5.0	4.9	4.7
T. repens (Ladino white)	4.8	4.8	4.9	5.0	4.9	4.8
T. semipilosum (Kenya white)	4.8	4.8	4.9	5.0	5.0	4.9
T. pratense (Pennscot red)	4.9	4.9	4.9	5.0	4.9	4.9
T. pratense (Mammoth red)	4.9	4.8	4.9	5.0	4.9	4.9
Rhizobia alone (no plants)	4.5	4.5	4.5	4.5	4.5	

The pH was measured initially and after 2 weeks. Results are the mean of 3 replicates.

TABLE 18. Nodulation of clover varieties inoculated with rhizobia strains and grown in liquid media at different pH levels.

Clover varieties	*Rhizobium trifolii* strains														
	T_1			T_1V_1			T_1P_2			$P_{1:1}$			$P_{1:2}$		
	4.5*	5.0*	6.0*	4.5	5.0	6.0	4.5	5.0	6.0	4.5	5.0	6.0	4.5	5.0	6.0
T. fragiferum (strawberry white)	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+
T. repens (Ladino white)	-	+	+	-	-	+	-	+	+	-	+	+	-	+	+
T. semipilosum (Kenya white)	-	+	+	-	-	+	-	+	+	-	-	+	-	-	+
T. pratense (Pennscot red)	-	+	+	-	+	+	-	+	+	-	+	+	-	-	+
T. pratense (Mammoth red)	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+

\+ Presence of nodule(s) in any plant of the three replicates. Each replicate had three plants per culture tube.

\- Absence of nodule(s) from all plants in the three replicates.

* The pH values.

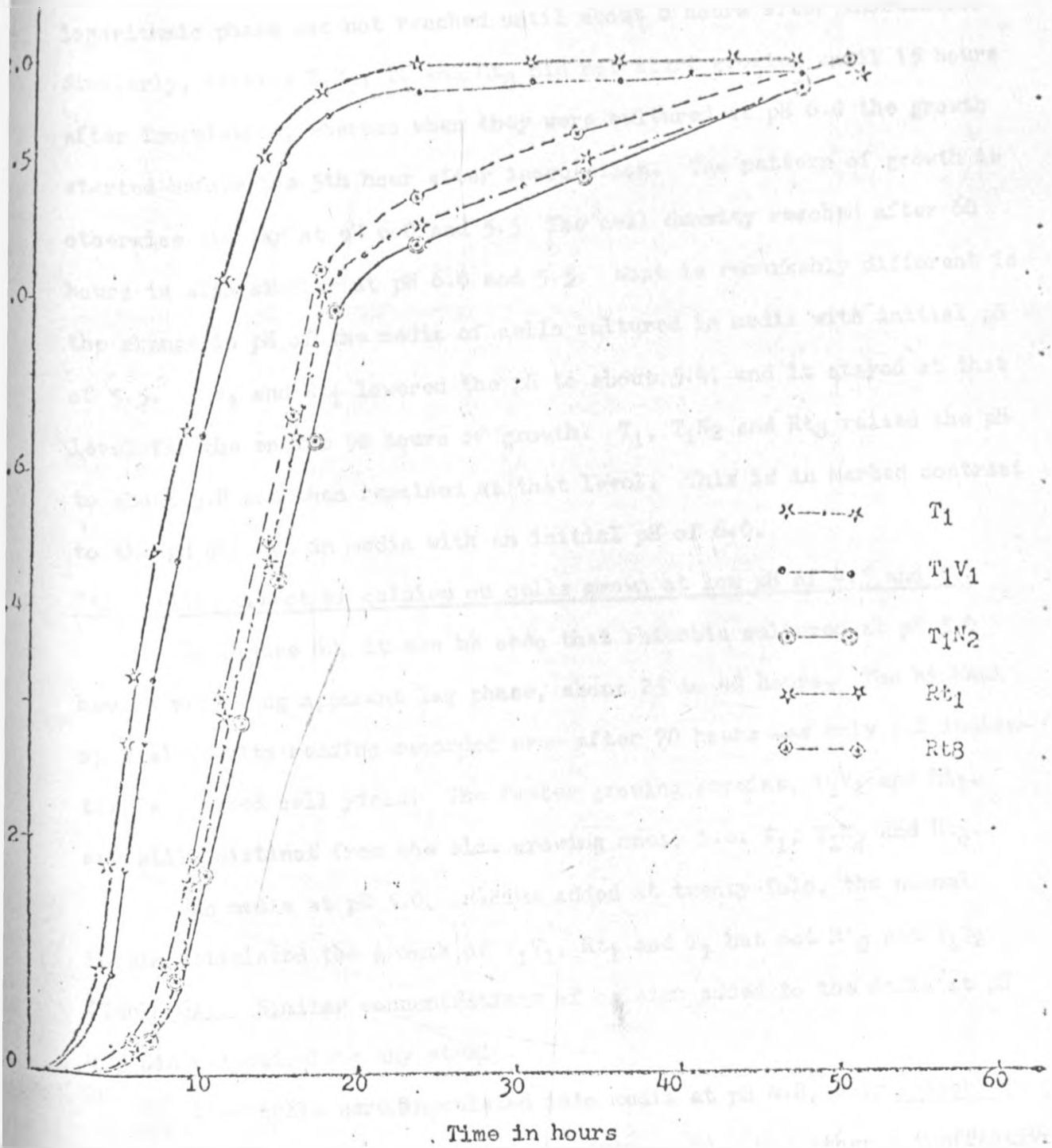


FIG. 2. Growth of Rhizobium trifolii strains in YEM broth and phthalate buffer at an initial pH of 6.0.

logarithmic phase was not reached until about 8 hours after inoculation. Similarly, strains T_1N_2, T_1 and Rt_8 did not start growing until 15 hours after inoculation, whereas when they were cultured at pH 6.0 the growth started before the 5th hour after inoculation. The pattern of growth is otherwise similar at pH 6.0 and 5.5 The cell density reached after 60 hours is also similar at pH 6.0 and 5.5. What is remarkably different is the change in pH of the media of cells cultured in media with initial pH of 5.5. T_1V_1 and Rt_1 lowered the pH to about 5.4, and it stayed at that level for the entire 90 hours of growth. T_1, T_1N_2 and Rt_8 raised the pH to about 5.8 and then remained at that level. This is in marked contrast to the pH changes in media with an initial pH of 6.0.

Stimulating effect of calcium on cells grown at low pH of 4.5 and 5.0

In Figure 6B, it can be seen that rhizobia cultured at pH 5.0 have a very long apparent lag phase, about 25 to 40 hours. The highest optical density reading recorded even after 70 hours was only 1.2 indicating a reduced cell yield. The faster growing strains, T_1V_1 and Rt_1, are still distinct from the slow growing ones, i.e. T_1, T_1N_2 and Rt_8.

In media at pH 5.0, calcium added at twenty-fold, the normal levels stimulated the growth of T_1V_1, Rt_1 and T_1 but not Rt_8 and T_1N_2 (Table 6A). Similar concentrations of calcium added to the media at pH 4.5 did not stimulate any strain.

When cells were inoculated into media at pH 4.8, only Rhizobium trifolii strain T_1, T_1V_1 and Rt_8 grew (Figure 7). The other 2 ineffective mutants, T_1N_2 and Rt_1, did not grow. Corresponding changes in pH also are shown in Figure 7 and it can be seen that where the cells grew, there was a slight change of pH from the initial one. T_1V_1 is an exception, because, although it grew at the pH of 4.8, there was no change of pH of the media.

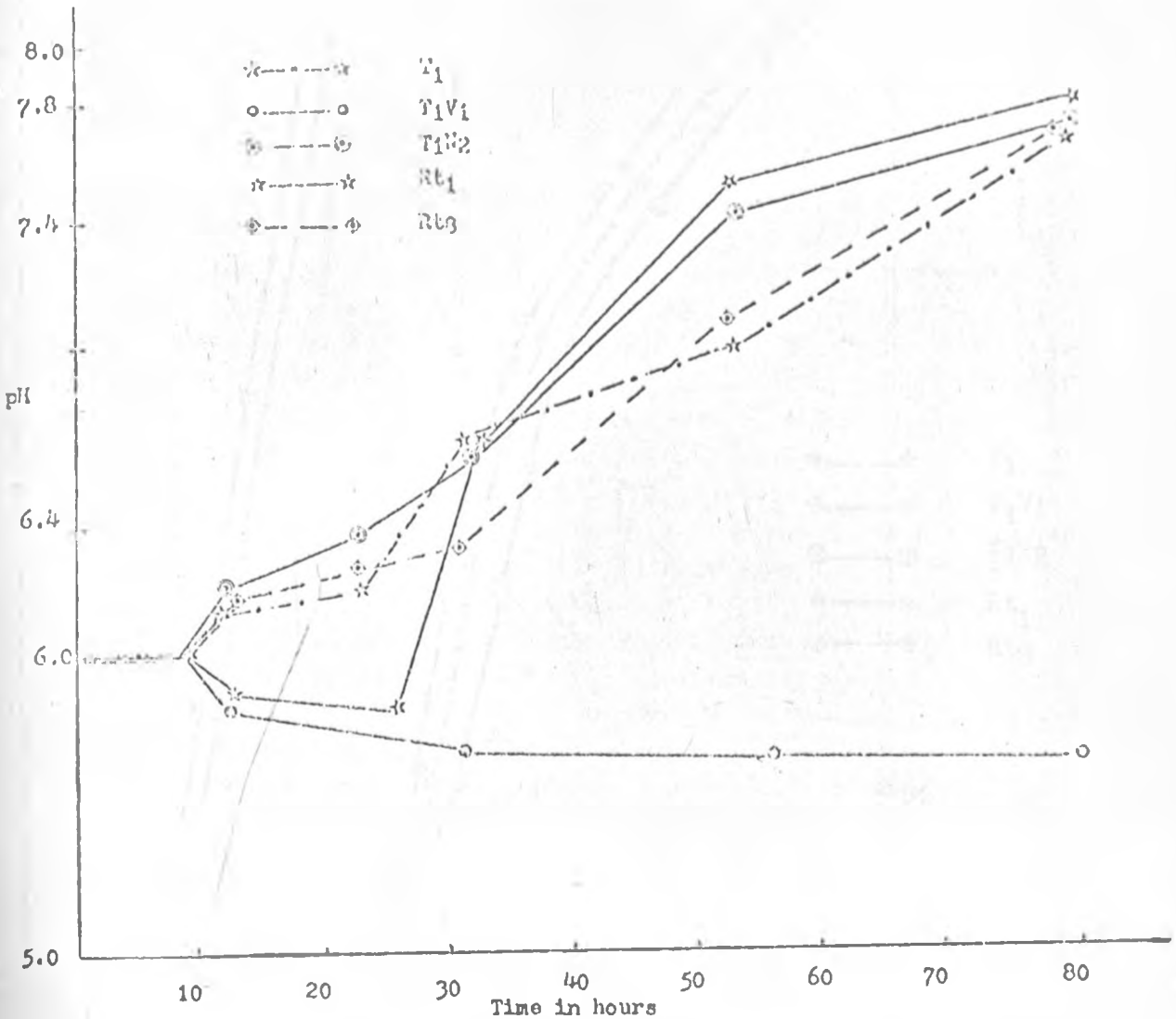


FIG. 3. The pH change of YEM broth media with an initial pH of 6.0 in which Rhizobium trifolii strains were cultured.

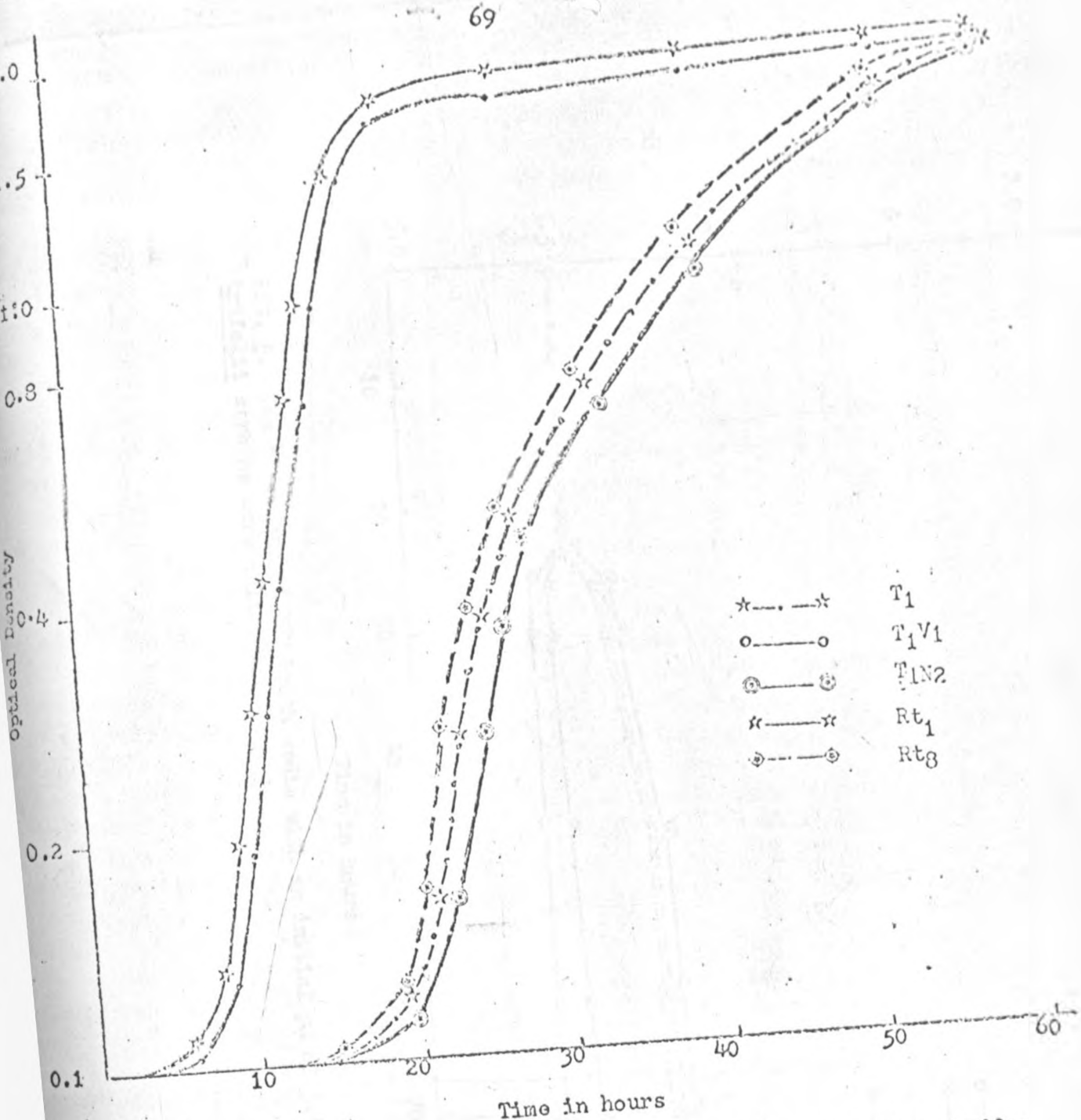


FIG. 4. Growth of Rhizobium trifolii in YEM broth and phthalate buffer at an initial pH of 5.5.

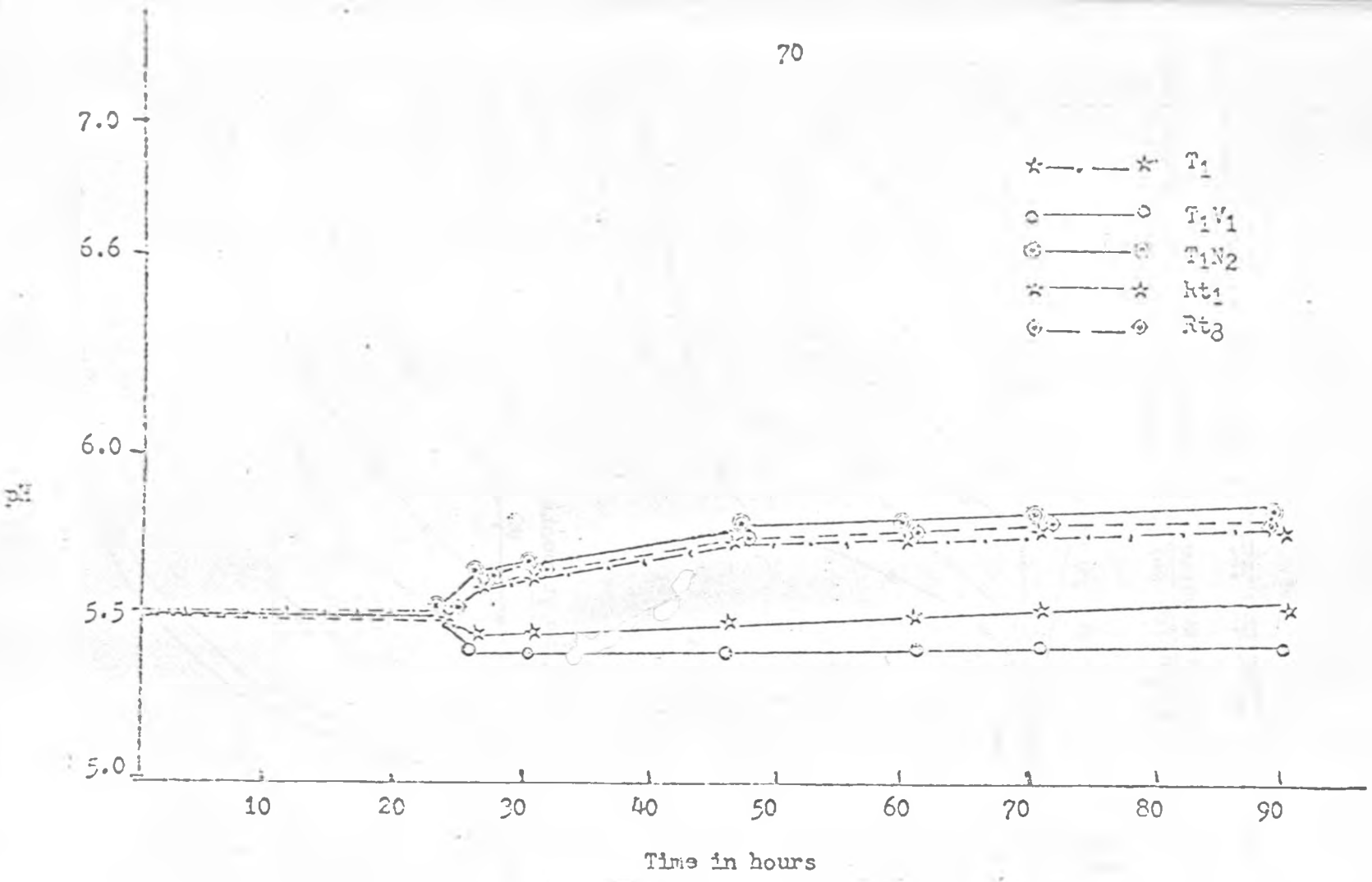
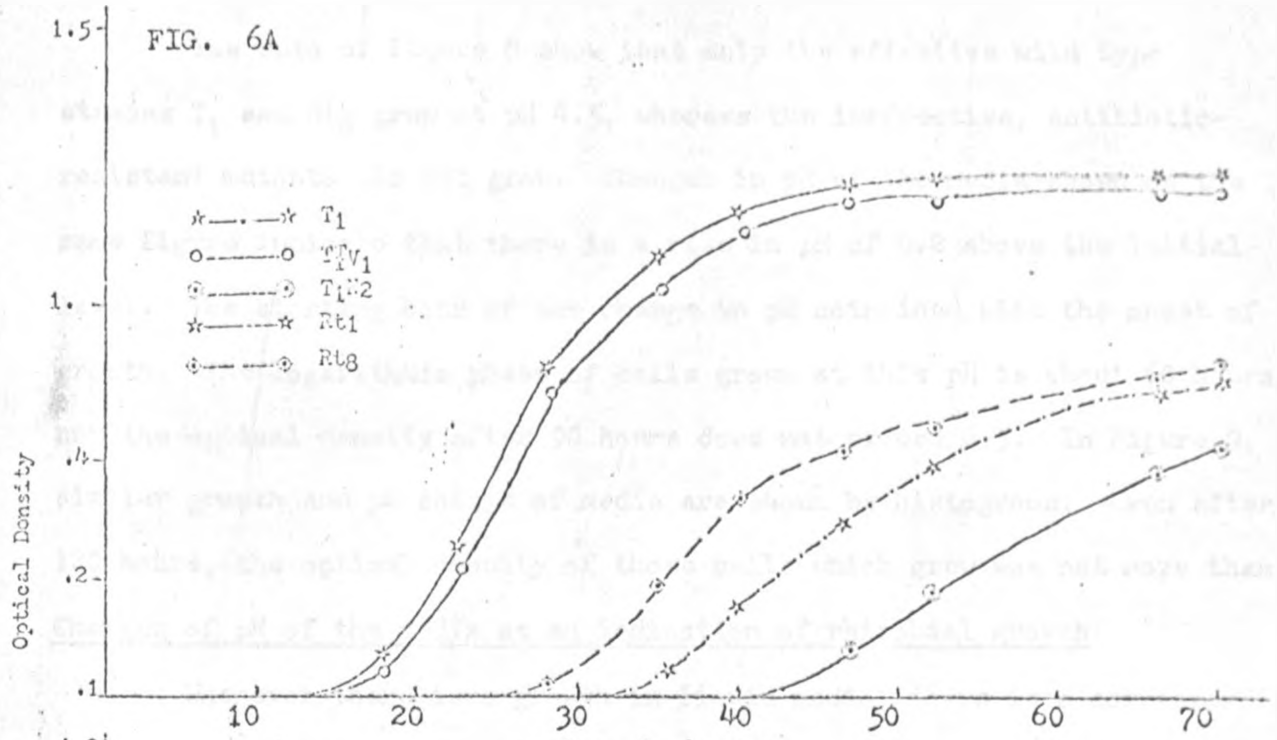


FIG. 5. The pH change of YEM broth media with an initial pH of 5.5 in which Rhizobium trifolii strains were cultured.



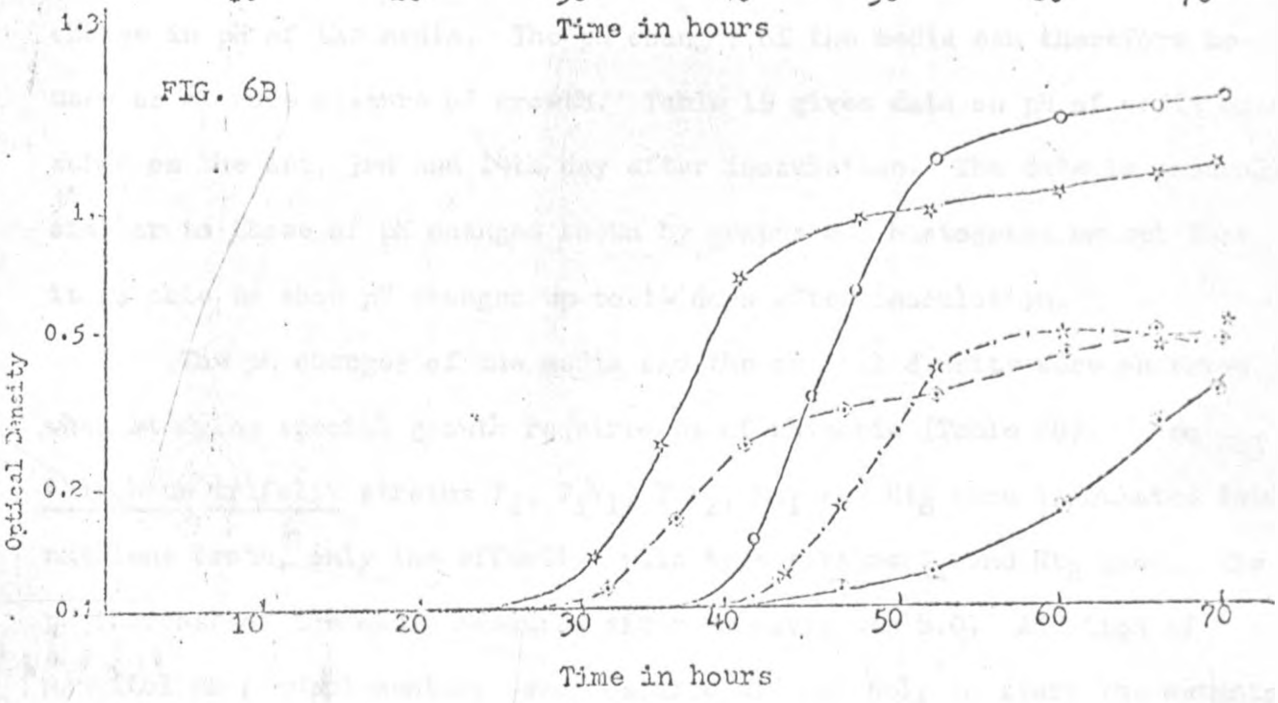


FIG. 6A. Growth of Rhizobium trifolii strains in YEM broth media at an initial pH of 5.0 supplemented with twenty-fold usual calcium levels.

FIG. 6B. Growth of Rhizobium trifolii strains in YEM broth media with an initial pH of 5.0.

The data of Figure 8 show that only the effective wild type strains T_1 and Rt_8 grow at pH 4.5, whereas the ineffective, antibiotic-resistant mutants did not grow. Changes in pH of the media shown on the same figure indicate that there is a rise in pH of 0.2 above the initial level. The starting hour of the change in pH coincided with the onset of growth. The logarithmic phase of cells grown at this pH is about 60 hours, and the optical density after 90 hours does not exceed 0.5. In Figure 9, similar growth and pH change of media are shown by histograms. Even after 120 hours, the optical density of those cells which grew was not more than 0.4.

Changes of pH of the media as an indication of rhizobial growth

Whenever there is a growth in liquid media, there is a corresponding change in pH of the media. The pH changes of the media can therefore be used as a crude measure of growth. Table 19 gives data on pH of media measured on the 1st, 3rd and 14th day after inoculation. The data is generally similar to those of pH changes shown by graphs and histograms except that it is able to show pH changes up to 14 days after inoculation.

The pH changes of the media and the optical density were observed when studying special growth requirement of rhizobia (Table 20). When Rhizobium trifolii strains T_1, T_1V_1, T_1N_2, Rt_1 and Rt_8 were inoculated into nutrient broth, only the effective wild type strains T_1 and Rt_8 grew. The pH increase of the media measured after 48 hours was 8.0. Addition of mannitol as a supplementary carbon source did not help to start the mutants growing. This showed that carbon source was not the limiting factor. When nutrient broth was supplemented with yeast extract, growth was abundant and rapid. Likewise, supplementation of the nutrient broth with casamino acids resulted in good growth and no prolonged lag phase. In all cases where growth occurred, the changes in pH were marked, always tending to the alkaline side.

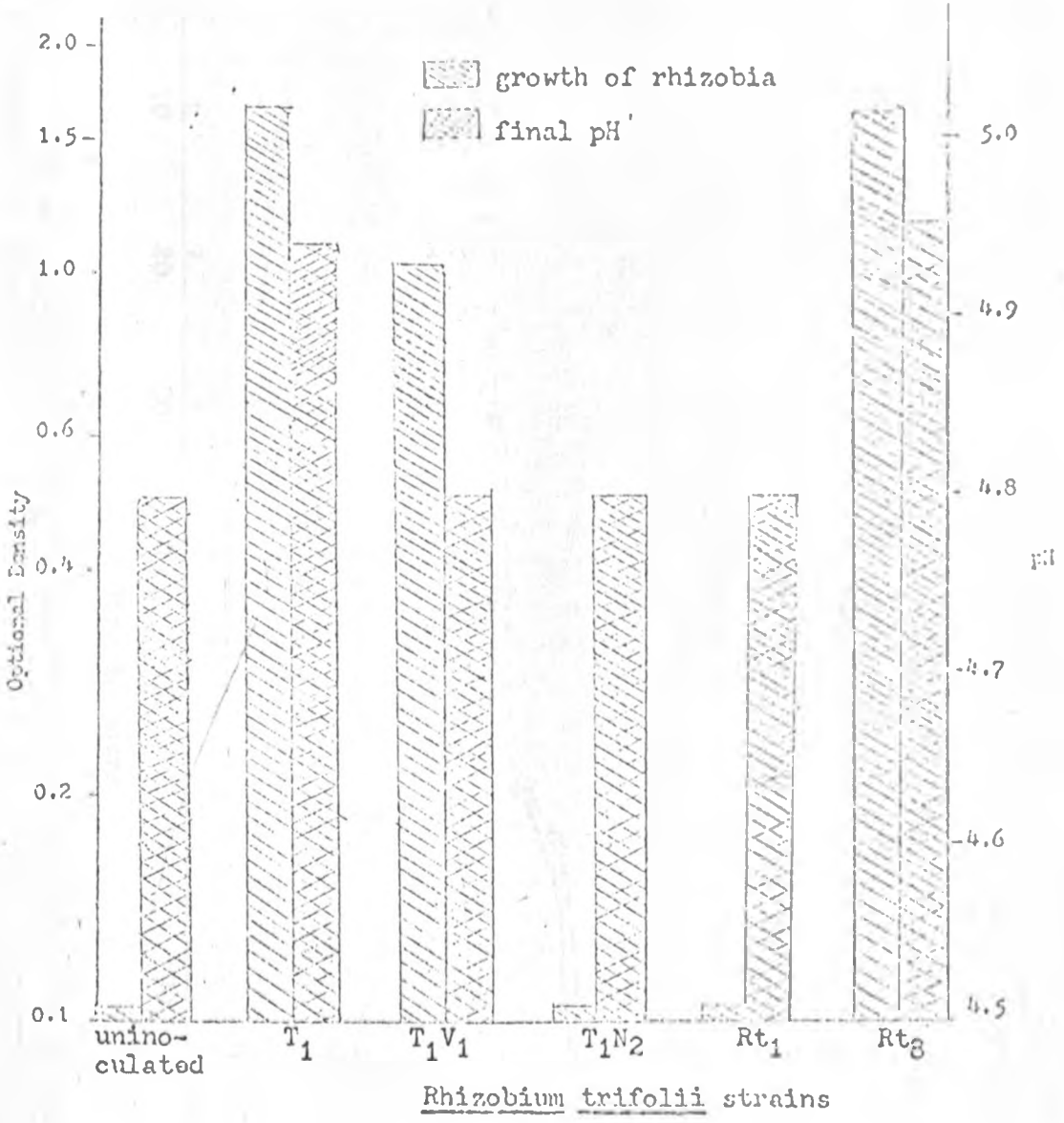


FIG. 7. Growth of Rhizobium trifolii strains in YEM broth with an initial pH of 4.8 and changes of pH in the media measured after 90 hours.

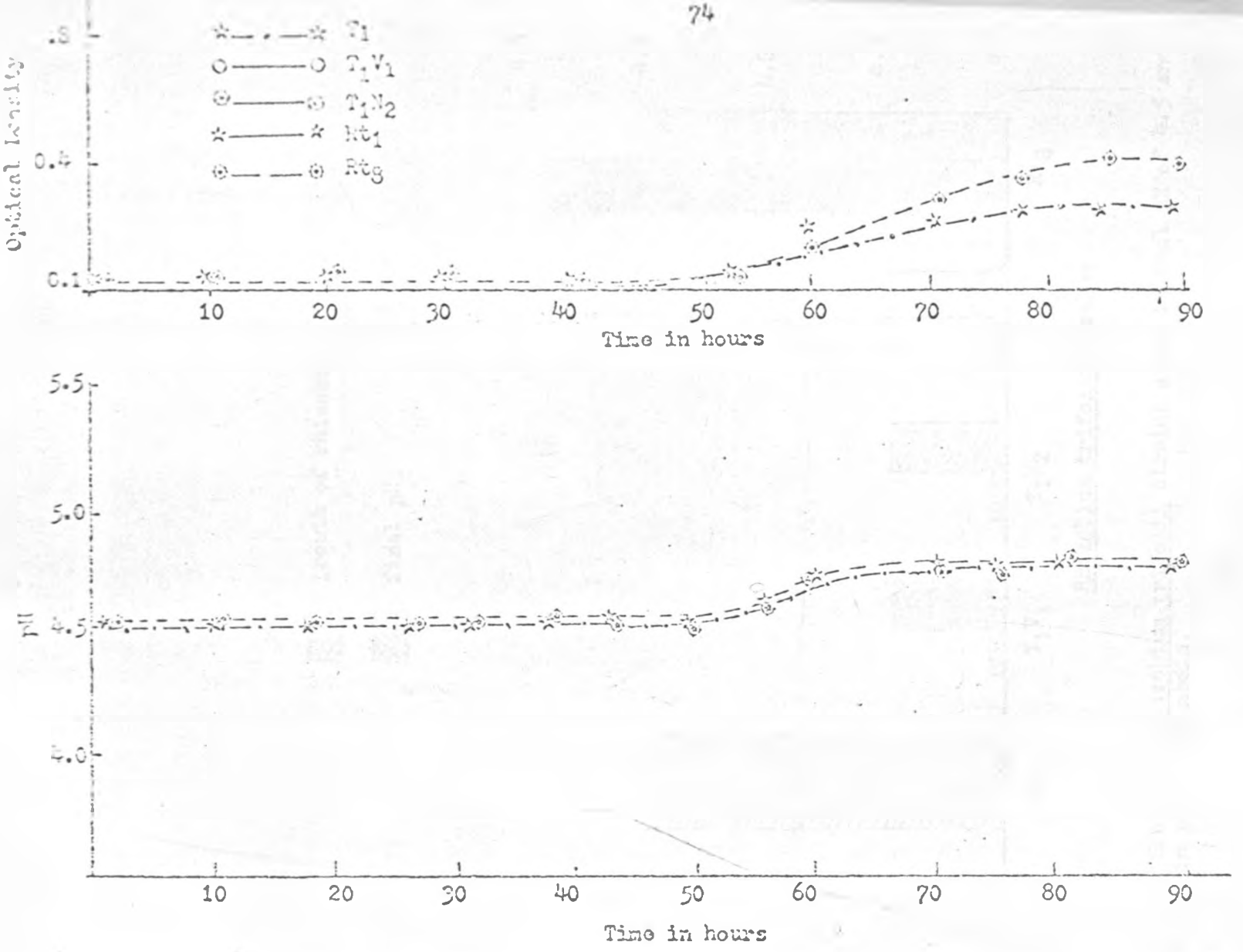


FIG. 8. Growth of Rhizobium trifolii strains in YEM broth media with an initial pH of 4.5 and pH changes in the media.

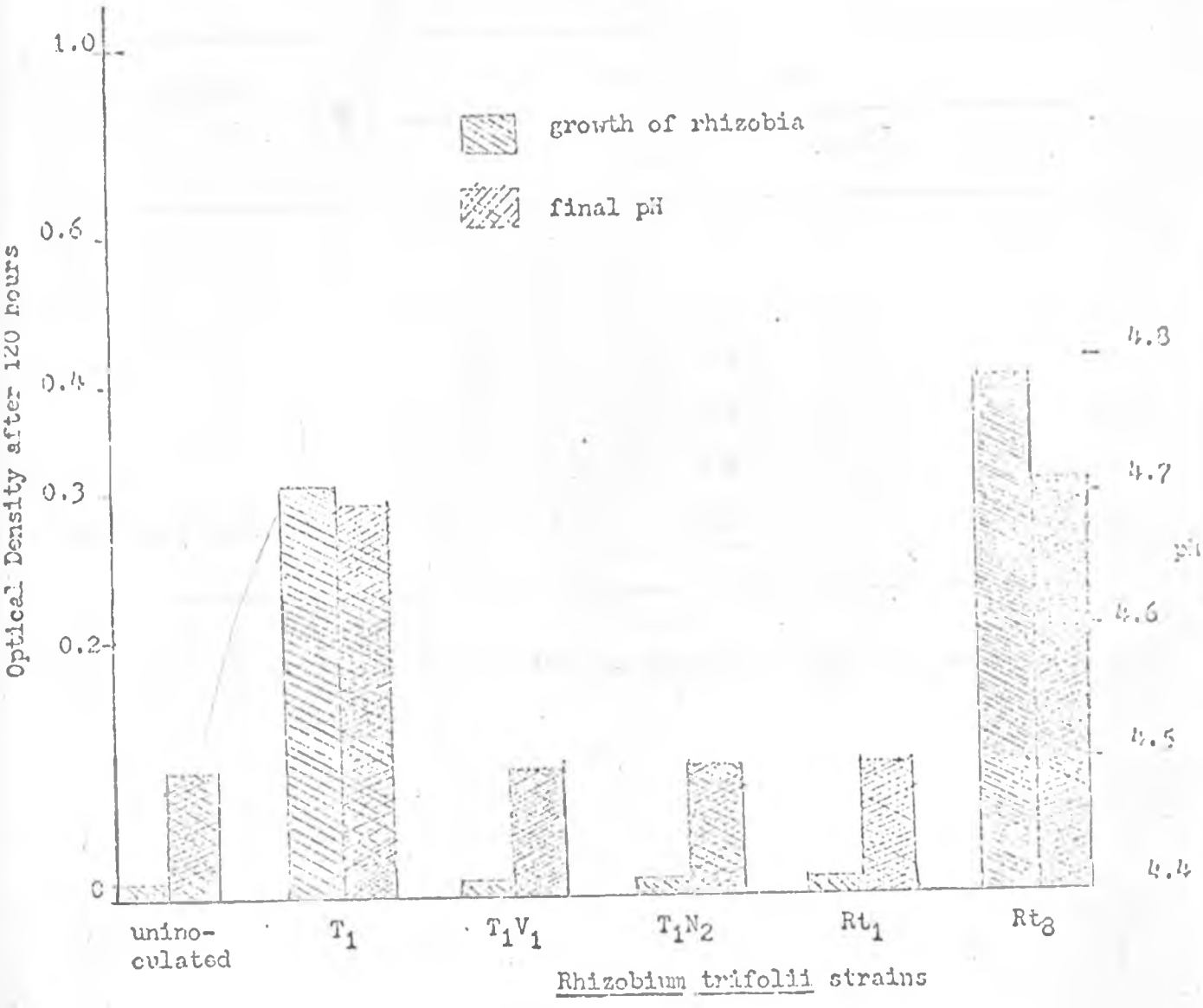


FIG. 9. Growth of Rhizobium trifolii strains at an initial pH of 4.5 and changes in pH of the media.

TABLE 19. The pH change of YEM broth as affected by growth of Rhizobium trifolii strains

Rhizobium trifolii	Days after inoculation		
	1	3	14
T_1	6.2	7.8	8.6
T_1V_1	5.7	6.7	8.0
T_1N_2	6.0	8.0	8.3
Rt_1	5.8	6.2	8.0
Rt_8	6.3	7.8	8.5
Uninoculated media	6.0	6.0	6.0

Initial pH of all media was 6.0. Potassium phthalate buffer was used.

Effect of pH on generation time, cell dry weight, protein content, and catalase activity

It has been shown in Figure 2 and Figure 4 that growth of the rhizobia studied did not differ much between pH 6.0 and 5.5. The generation time at those pH values are not too different either (Table 21). The generation time at pH 5.0 was not calculated since at that pH the growth was affected so much that any attempt to calculate generation time would have led to serious errors.

Table 22 presents data showing cell dry weight and protein content of rhizobia grown in axenic cultures at pH 6.0 and 5.0. There is no difference in the protein content of cells cultured at those 2 pH levels. Cells grown at pH 6.0 seem to have a higher dry weight than those grown at pH 5.0, but the difference is quite small. It should be pointed out, however, that although all the cell suspension used had the same final optical density (1.0), the cells grown at pH 5.0 did not attain that level of growth until 90 hours from time of inoculation. The same cells grown at pH 6.0 attained an optical density of 1.0 after 24 hours. The results of protein determination obtained by the tyrosine method are lower than those obtained by the micro Kjeldahl method. Probably cells were not completely sonified in the tyrosine method.

A study of catalase activity shown in (Table 23) showed that although some strains did not grow at pH 4.5, they still exhibited some catalase activity. Catalase activity was best at pH 8.0, on the alkaline side. When the pH was reduced to 2.0, for $\frac{1}{2}$ hour and then raised to 11 and then catalase activity was tested, it was found out the cells had catalase activity. From these results, it can be inferred that the effect of low pH is only bacteriostatic and not lethal.

TABLE 20. Growth of rhizobia in various media

Medium	*Rhizobium trifolii* strains										Uninoculated media	
	T_1		T_1V_1		T_1N_2		Rt_1		Rt_3			
	O.D.	pH	O.D.	pH	O.D	pH	O.D.	pH	O.D.	pH	O.D.	pH
Nutrient Broth	0.65	8.0	0	6.8	0	6.8	0	6.8	0.57	8.2	0	6.8
Nutrient Broth + Mannitol	0.91	8.0	0	6.8	0	6.8	0	6.8	0.83	8.2	0	6.8
Nutrient Broth + Yeast extract	1.35	8.0	1.34	7.8	1.31	8.4	1.33	8.0	1.31	7.8	0	6.8
Nutrient Broth + Casamino acids	1.24	8.2	1.37	8.0	1.16	8.1	1.18	8.2	1.22	8.2	0	6.8

O.D. = Optical Density and pH measurements were read 48 hours after inoculation.

Initial pH of the media was 6.8.

TABLE 21. Generation time of *Rhizobium trifolii* strains cultured in YEM at pH 5.5 and 6.0

Rhizobium trifolii strains	Generation time in hours	
	pH 6.0	pH 5.5
T_1	3.6	5.6
T_1V_1	3.2	3.0
T_1N_2	3.7	6.2
Rt_1	3.1	3.0
Rt_8	3.6	5.8

TABLE 22. Cell dry weight and protein content of rhizobia grown at pH 6.0 and 5.0

Rhizobium trifolii strains	pH 6.0		pH 5.0		* Tyrosine Method
	Dry+	Protein	Dry	Protein	Protein
T_1	1.9	0.19	1.7	0.16	0.14
T_1V_1	1.5	0.25	1.2	0.22	0.10
T_1W_2	1.8	0.25	1.3	0.25	0.25
Rt_1	1.2	0.13	1.1	0.25	0.17
Rt_8	1.8	0.22		0.25	0.13

All cell suspension used had an optical density of 1.0 in the case of bacteria grown at pH 6.0 and 5.0.

* Cells had an optical density of 0.5 but the results were adjusted by a dilution factor to an optical density of 1.0. The cells were cultured at pH 6.8

+ Dry weight of cells expressed in mg. per ml.

Varying the concentration of initial inoculum as an attempt to alleviate the low pH effect

Results of the experiment reported in Figure 10 aimed at testing whether high concentration of initial inoculum would overcome the effect of low pH. Figure 10 shows that this is not the case. No growth was obtained with an initial cell concentration of 1×10^3 cells per ml. Any concentration above 1×10^3 cells per ml. gave good growth. The highest concentration of 10×10^{10} cells per ml. did not give the best growth. It is also clear that once growth was initiated, the original concentration of inoculum did not affect final cell yield.

Enzymatic breakdown of pectin

The assay for enzymatic breakdown of pectin gave results which showed very little breakdown of pectin (Table 24). The flow time in seconds measured viscometrically indicates that the filtrate obtained from plant culture solution of uninoculated seedlings had the same activity as those of the inoculated ones. Activity of the enzyme obtained from plant culture solution filtrates of 1-week and 2-week old seedlings were not different. Similarly, enzyme activity of plant culture solution obtained from seedlings grown at pH 6.0 and pH 5.0 was similar to those of uninoculated seedlings. The culture filtrate of rhizobia grown in pure culture showed no enzyme activity at all. This was to be expected since the enzyme is an induced one and is produced only in the rhizosphere of inoculated seedlings and not by bacteria alone (37).

TABLE 23. Polygalacturonase activity of clover seedlings inoculated with *Rhizobium trifolii* strain T_1

Time + in hour(s)	pH 6.0		pH 5.0		filtrate of plant culture solution from uninoculated plants	Culture filtrate of *Rhizobium*
	One-week old seedlings	Two-week old seedlings	One-week old seedlings	Two-week old seedlings		
0	11.9*	11.4	12.0	11.6	11.7	11.9
2	11.9	11.4	12.0	11.6	11.7	11.9
4	11.8	11.4	12.0	11.6	11.7	11.9
6	11.7	11.3	12.0	11.5	11.7	11.9
12	11.7	11.3	12.0	11.5	11.6	11.8
15	11.7	11.3	12.0	11.5	11.7	11.9
18	11.6	11.3	12.0	11.5	11.7	11.9
22	11.6	11.2	11.9	11.5	11.7	11.8
24	11.6	11.2	11.9	11.4	11.7	11.8

* Flow time in seconds as measured by viscometers. Results are mean of four replicates. Deionized distilled water had a water efflux of eleven seconds.

+ Time in hours when polygalacturonase activity was assayed.

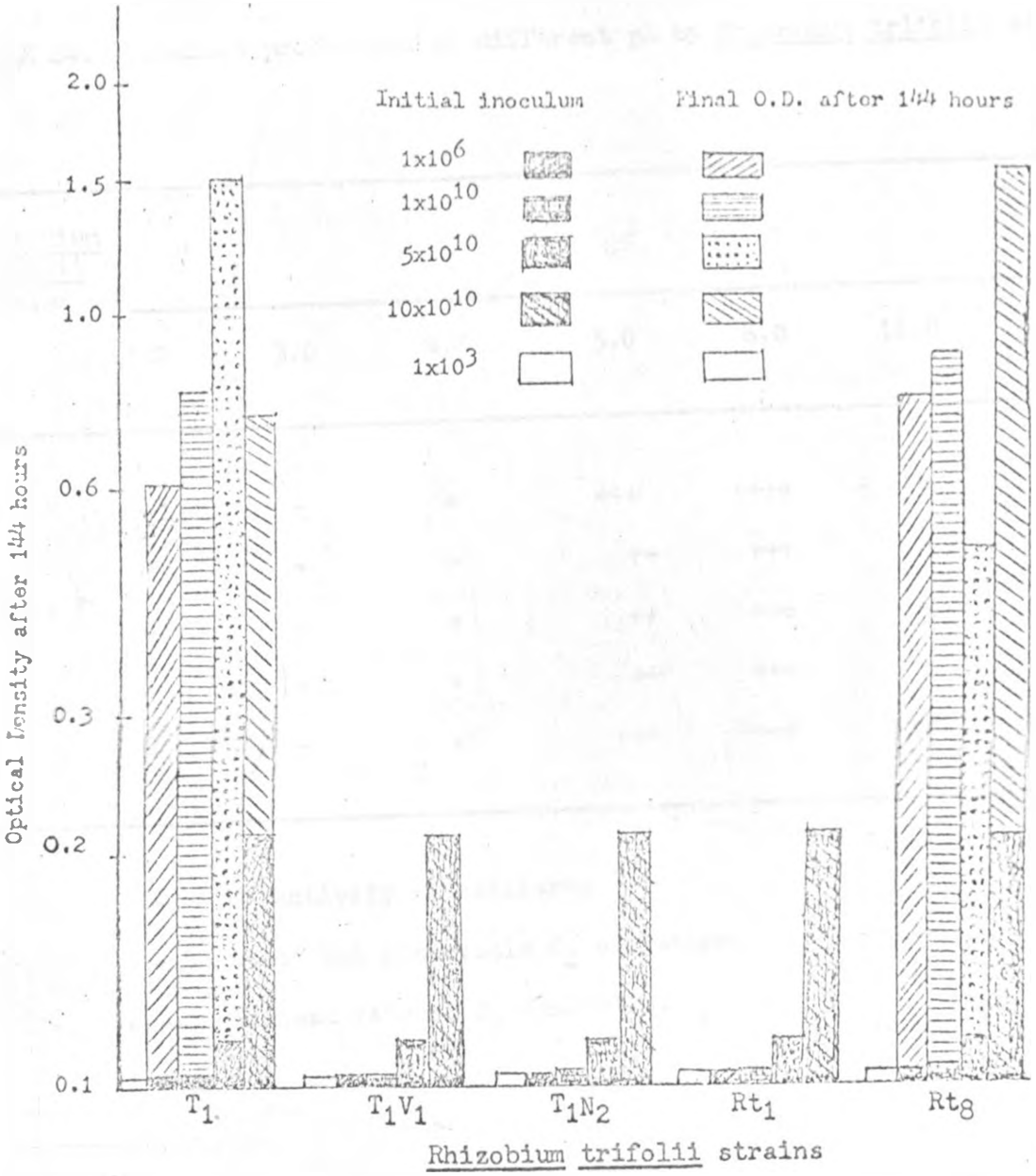


FIG. 10. Effect of varying ıe size of initial inoculum on growth of Rhizobium trifolii strain. cultured at an initial pH of 4.5.

TABLE 24. Catalase production at different pH by *Rhizobium trifolii* strains

Rhizobium trifolii strains	pH						
	2.0	3.0	4.5	5.0	8.0	11.0	2.0 then 11.0
T_1	-	-	+	+++	++++	++	++
T_1V_1	-	-	+	++	+++	+	+
T_1N_2	-	-	+	++	+++	+	+
Rt_1	-	-	+	++	+++	+	+
Rt_8	-	-	+	+++	++++	++	++

\- No activity of catalase.

\+ Least but observable O_2 evolution.

++++ Highest rate of O_2 evolution.

DISCUSSION

The fact that resistance to antibiotics in Rhizobium may be accompanied by the organism's inability to nodulate its host plant, as well as by ineffectiveness, tends to indicate that the antibiotic is not a specific selective agent for either property.

The polypeptide antibiotics streptomycin, neomycin and viomycin are compounds active on bacterium's surface. Due to free amino groups in the molecules, they tend to have a high net negative charge. These antibiotics are known to cause extensive damage to the cell membrane (47). In low concentrations(below 300 ppm), they may cause external breakdown of ribosomal RNA. In higher concentrations, these antibiotics are capable of binding onto the RNA molecule, causing stabilization and changes in activity of the ribosomes (22). Since they interact in some way with the ribosomes, but may not directly affect the DNA, they may somehow alter the translocation of the genetic sequence from DNA to the RNA or of the RNA into the appropriate protein molecule.

There is no direct evidence that these antibiotics are mutagenic, although Jordan, Yamamura and Mckague (22) showed that single step viomycin resistance resulted in ineffectiveness of R. meliloti from parent effective strain. They concluded that ineffectiveness is almost certainly related to a single genetic change in R. meliloti. They also postulated that ineffectiveness due to viomycin resistance, caused marked deformation of growing rhizobia and suggested that ineffectiveness is caused by a mutation to an altered wall structure. Viomycin resistance is in some way related to a block in bacteroid formation and inhibition of protein synthesis.

An alteration in cell permeability is perhaps another way the relation between antibiotic resistance and ineffectiveness can be explained. The development of resistance to these antibiotics might entail changes in membrane

tructure, changes which might also affect the effectiveness of bacteria. he changes may be changes in permeability, in some essential enzyme on the embrane or in a step in electron transport or other energy-transfer mechanism ssociated with the membrane. Any such change could conceivably result in a hange in the effective capacity of the bacteria.

The role of actinomycetes (viomycin and neomycin are all produced by Streptomyces spp) and other soil-inhabiting microbial antagonists in the legume rhizosphere should be considered not only in relation to rhizobial competition or survival but also from the standpoint of possibly altering the genetic level of ability to participate in symbiosis. Holland and Parker (18) has shown that the problem of clover establishment on virgin soils appears to be caused by antibiotic-producing fungi which proliferate on the organic debris remaining after the original vegetation has been removed.

One of the aims of this study was to look for a Rhizobium trifolii strain which would nodulate one or several clover varieties and yet fail to nodulate other clover varieties. Such a strain, if found, might have given some insight of specificity within a single inoculation group of Trifolium spp. This search was unsuccessful with the combination of 9 Rhizobium trifolii strains and 6 Trifolium species used. Perhaps with a greater number of Rhizobium strains and Trifolium species, a non-nodulating combination(s) might have been encountered. It was noted, however, that Trifolium semipilosum (Kenya white clover) inoculated with Rhizobium Trifolii strains T_1, T_1V_1, T_1N_2, Rt_1 and Rt_3 grown in petri dishes and plant culture tubes did not form nodules at all times. Sometimes plants in only one of the three replicates in any one treatment formed nodules. Since there was no consistency in failure of nodule formation, it was concluded that poor nodulation in Kenya white clover could be due to unfavorable conditions.

Kenya white clover is a cool, high altitude adapted variety which grows at temperatures of about 10 to 16°C. throughout the year. Temperatures in the growth chambers were always 25 ± 2°C.

It had been reported (in Results), that Kenya white clover grown in petri dishes and plant culture tubes sometimes died after or within 2 weeks. Death of the seedlings can be accounted for by poor environmental conditions which could interfere with nodulation. There was no nodulation problem or death of seedlings when Kenya white clover was grown in pots or Leonard jars in either greenhouse or growth chamber. Since sand was used in the pots and in the Leonard jars, it can be speculated that sand provided a more stable root environment than agar or liquid media, thus permitting less fluctuation in physical factors.

It was also pointed out that few nodules formed on Kenya white clover in petri dishes and in plant culture tubes. This would be misleading if Kenya white clover were rated by criteria of nodule number as forming effective sysmbiosis. Few nodules can also be accounted for by improper environmental conditions. Rhizobium trifolii strains S_2, H_2, W_2 and R_2, when tested for their nodulation ability proved to be good nodule-formers, especially in petri dishes. Since these same strains showed excellent growth in YEM agar, it was inferred that their vigor was possibly due to the fact that they had just recently been isolated and had not suffered degeneration due to transfer through mineral stock media or storage.

Many methods are available for testing nodulation and nitrogen fixation. The techniques used in this survey make it possible to compare the various methods of testing for nodulation. Fahraeus' technique is a convenient and simple method for observing infection thread and nodule development. It is convenient in that the seedlings attached to the glass slide can be

ily removed, placed under a light microscope for examination, and can
ned to the plant culture tube without contamination. By this method,
ossible to follow nodule development from as early as 5 days, and by
day most plants could be scored for presence or absence of nodules.
nique was also used to estimate an increase in numbers of rhizobia
legume rehizosphere, and the numbers obtained (Tables 15 and 16), are
ble to those reported by others (40), (41). It was also found to be
e for detecting nodulation in liquid culture at different pH values.
hnique was appropriate for handling small clover and alfalfa seed-
ut not big varieties of clover plants such as T. subterranean. One
weaknesses of the technique is that the glass slide is small and if
are kept for more than 2 weeks, the roots grow beyond the glass slide
ie bottom of the tube. This overgrowth of plant roots renders frequent
ig cumbersome. The plant culture tube being short, if the plants
ft growing for a long time, they become limited in vertical space for
growth, since the plant culture tubes were short. There is no device for
ting the seedlings' root from light. Diffusion of CO_2 which has been
o be a limiting factor to plants grown in test tubes Gibson (personal
ications), was not found to be a problem.

The plant culture tube, which is a simplified form of Fahraeus'
ique can cope with taller plants. Since the tubes are longer, gaseous
ssion is enhanced, plants can grow much taller and therefore can be
for a longer time. When plants were grown on colorless agar slants,
re tubes, on slants, culture tubes, no support such as glass slides was
sary. Nodulation was good, and differences in effectiveness and ineffec-
oss could be detected by the end of one month. The system requires
e watering, and the nodules can be examined without the plants being
ed, without or by the aid of a hand lens. Since, once set up, the plants

are not handled once they have been mounted aseptic techniques can be adhered to much better.

The petri dish method was another good technique for testing nodulation and nitrogen fixation by clovers. When the dishes were covered with polythene, moisture control was good, and no watering was desirable for the entire testing period. The testing period could extend as long as 1½ months. Like the other 2 methods already described, many plants can be handled in a small growth chamber. Vertical and horizontal limitation of space in petri dishes however, suppresses full plant growth. Carbon dioxide and O_2 diffussion is likely to be more limiting than in the plant culture tubes, but it is possible to observe effective and ineffectivoness within 1 month from the time of inoculation. Nodules can easlily be examined and counted either visually or with the aid of a dissecting microscope. Results of dry weight and nitrogen determinations did not clearly distinguish between effectively and ineffectively nodulated plants. This is probably due to the fact that the plants did not grow large enough to deplete their cotyledonous nitrogen. Also, since the larger the plant, the higher the nitrogen content, the plants grown in petri dishes were too small in size to contain high amount of nitrogen, which could show significant differences.

Clay pots with sand was a much better method of testing for nodulation and nitrogen fixation. In the growth chamber, when space is limiting, chances of contamination are likely to be high, especially if the pots are too close together. Chance of contamination are increased because the pots have wide open tops. In the greenhouse, contamination can be reduced by wider spacing of pots. If autoclaving is not thorough, undesired rhizobia are likely to cause contamination.

Leonard jars have become a standard method for testing nodulation and

nitrogen fixation. The assembly is economical in water use in that it is self-irrigating and needs watering only once a week. Bacterial contamination is greatly reduced, the jars occupies less space and give the roots more depth for growth and development. It should be pointed out that salts tend to accumulate at the top of the sand, but the salt can be washed down with frequent watering.

In both pots and Leonard jars in the greenhouse, algae developed on the surface of the sand. The sand was analyzed for nitrogen before and after each testing for nodulation or nitrogen fixation, and it was found out that the increase in nitrogen in the sand was in the order of 0.005 mg. per 1 g. of sand. This is a small contribution of nitrogen by the algae, and is not likely to interfere with estimates of symbiotic nitrogen fixation. Plants grown in sand using pots or Leonard jars in growth chambers gave as close dry weight and nitrogen content as those grown in greenhouse. However, no known greenhouse or laboratory procedures can be used to predict the type of field results that may be expected.

The 2 effective Rhizobium trifolii strains, T_1 and Rt_8, were not equally effective on all clover varieties. This study showed that Rhizobium trifolii strain T_1, which was known to be effective on red clover, was also effective on white clover. Similarly, Rhizobium trifolii strain Rt_8 was not only effective on white clover but also on red clover. This finding supports the observations of Roberts and Olson (42) that Mammoth red clover combined with 5 cultures of Rhizobium trifolii differed in relative efficiency in nitrogen fixation. It is also in agreement with studies of Erdman (12), who studied the efficiency of 15 strains of Rhizobium trifolii obtained from 10 species of Trifolium. Each strain was inoculated into jars containing seedlings of the following clover species: Trifolium alexandrum, T. fragiferum,

T. subterraneum and T. resupinatum. No one bacterial strain was equally effective with each of the 4 plant species. The variation of effectiveness was considerable.

The number of nodules formed was not a reliable indication of the amount of nitrogen, nor of the total yield of the plant. Erdman concluded that maximum growth and fixation depends upon the use of an effective strain. Those varieties on which strains T_1 and Rt_8 were effective, strain T_1 proved effective in association with that plant species and ineffective with red clover or white clover. This strain variation in effectiveness, which can be attributed to specific host plant reactions is distinct from that displayed by strains effective or partly effective upon one particular host, a variation attributable to virulence. This study also revealed, as summarized in Table 10, that strain T_1 and Rt_8 were effective on T. fragiferum (strawberry white clover), T. repens (Ladino white clover), and T. pratense (Pennscot and Mammoth red clovers) varieties from New York State. The same strains were ineffective on T. fragiferum, T. repens, T. subterraneum varieties from Israel and a T. semipilosum (Kenya white clover) a variety from Kenya. It should be noted that New York State, Israel and Kenya, lie in the temperate, sub-tropical and tropical regions of the world respectively.

Weir (56) who compared the performance of R. meliloti containing commercial inoculum originating in U.S.A. and the Union of South Africa, found that the ratio of ineffective to effective types was highest in those plants inoculated with the South African inoculum. Weir attributed the relative lack of success of the American strain to a lack of adaptation between varieties of plant and nodule bacteria from South Africa. Some adaptation to local conditions appeared to have occurred over the 6-year period during which the rhizobia had existed in the local soil. Erdman (11) gave evidence that a soil in

Alabama contained native nodule bacteria which could fix nitrogen in adapted soybeans but were incapable of fixing nitrogen in unadapted varieties of the plant. Effective strains of soybean bacteria isolated from commerical soybean vaieties in the corn belt are not generally effective on Southern soybean varieties. Norris (38) found that African clovers caused effective nodulation on African clovers, whereas the European clovers formed ineffective nodules on African clovers. He also showed that the European clover rhizobia failed to cause nodulation on the African Trifolium species. Evidence provided in the present study, together with those quoted, can only lead one to suggest that the development of commercial inoculants especially suited for legumes to be grown on particular soil types is necessary to give optimum results. It would appear that introduction of a new Trifolium species into another country is of little potential value unless suitable rhizobia, which are symbiotically nitrogen fixing in the tropics or sub-tropic are obtainable. These can usually be obtained in the native environment.

Development of an inoculum industry based on local isolates tested for effectiveness on particular legumes would be proposed if inoculation continues to be part of agronomic recommendation in legume culture. Introduction of new legumes should be coupled with their corresponding effective rhizobia, from the country of origin. Should the rhizobia fail to establish themselves on local soils, then the native isolates will have to be looked for.

The effectively nodulated clovers used in this study were tested for their relative effectiveness of nitrogen fixation. The results of Table 10 show that the white clovers fix more nitrogen per plant than red clovers. On the other hand, the red clovers, being larger than white clovers, attain a much greater dry weight than white clovers. In New York State, it is Pennscot and Mammoth red clovers that are recommended for use in pastures, yet they are the least nitrogen fixers per plant. Probably this recommendation is based on

their higher dry weight. If this be true, then there is no reason why white clovers should not be recommended on their ability to fix more nitrogen per plant.

Other parameters of rating ineffectiveness such as shoot to root ratio can be useful guides of effective or ineffective symbiosis. Whereas nodule numbers are not always a reliable indication of ineffectiveness, it would be interesting to know the ratio of effective to ineffective nodules on a given plant, a situation which normally prevails in the field where mixed nodulation is possible. In examining intact nodules, it was found that in some of the ineffective rhizobia-legume combinations, there were signs of a pink to reddish pigment. This could also be a misleading criteria of judging effectiveness.

Attempts to find physiological differences between effective and ineffective strains was not very successful. However, growth studies at different pH revealed some striking differences. At pH 6.0, growth of all the 5 strains studied in detail was good. It was apparent that the strains could be clearly separated into fast- and slow-growing types. The fast-growing types 1st produced acid, then alkali, whereas the slow-growing types produced alkali. The acids produced were not determined, but it can be speculated that these could be organic acids arising from mannitol and amino acids present in the yeast extract. Alkalinity, on the other hand, may be attributed to the production of NH_4^+, or basic amino acids. That fast-growing strains (e.g., Rt_1 and T_1V_1) produced acid(s) was also shown when the strains were grown at pH 5.5. The fast growing strains lowered the pH to 5.4, and the pH remained at that level for the rest of the incubation period. At pH of 5.5, the slow-growing strains (e.g., T_1, T_1N_2 and Rt_3), still produced alkali.

At pH 5.0, the apparent lag phase was long. In addition, the cells did not reach as high an optical density as those grown at pH 6.0 or 5.5. At pH 4.8, only T_1, T_1V_1 and Rt_8 grew, and at pH 4.5, only strains T_1 and Rt_8 grew. It is interesting to note that those strains that failed to grow at pH 4.5 were antibiotic-resistant, ineffective mutants. At pH 4.5, increasing the initial inoculum did not help to alleviate the effect of low pH. It is noteworthy too, that 2 of these strains (T_1V_1 and Rt_1), were acid producers and fast growers, and yet they are the ones which failed to grow in an acid media. It should also be pointed out that strains T_1 and Rt_8 were the slow growers, alkali producers, wild type, antibiotic susceptible and effective on red and white clovers. It was shown by Table 18, that the ineffective strains did not grow in nutrient broth but grew in yeast extract and also in casamino acids. This indicated that they required certain vitamins, most likely B vitamins usually supplied by yeast extract. From these observations it is likely that, there is an altered metabolism between the prototrophic parent strains and the auxotrophic antibiotic mutant strains. It is tempting to correlate ineffectiveness or effectiveness to low pH tolerance, but too few rhizobia strains were studied to allow for firm conclusions. However, Norris, (personal communication), suggested that rhizobia able to tolerate acidic conditions produce alkali, and those tolerating alkaline conditions produce acid.

It was found in this study that Ca stimulated growth at pH 5.0, but not at pH 4.5. Stimulation of growth by Ca at pH higher than 5.0 was not performed. This work, is in support of the results of Vincent (53), who reported Ca stimulation of rhizobia at pH 5.5. In contradiction, Norris (39), states that Rhizobium need Mg, but not Ca. Stimulation of cells at pH 5.0 by Ca may be due to the fact that Ca restores cation balance which would not

otherwise be upset at low pH of 5.0. By this same argument, Ca stimulation was not possible at pH 4.5, because at that pH, the upset of ionic balance was too great to be restored. Even at the pH of 4.5, the cells still showed catalase activity, although they were not actively growing.

Munns (37), has shown that the nodulation process in Medicago sativa is most sensitive to acid inhibition during the initiation of infection, at a time before the infection thread appears in the root hair. Once initiated, infections will develop and nodules will form despite a significant drop in pH. Thus the pH dependence of the whole nodulation process may be controlled by the pH dependence of one event early in infection. Although it is risky to extrapolate axenic culture studies to natural systems such as symbiosis, it is possible that growth of rhizobia is not adversely affected at pH 6.0 and 5.5. This is probably the reason why nodulation is good at those pH values. At pH 5.0, on the other hand, when the lag phase was extremely prolonged, growth rate and cell yield was low in pure culture and nodulation was poor. At pH 4.5, when there was no nodulation at all, three of the strains did not grow, and even those which grew did not do so until 60 hours after inoculation. Since nodule initiation is inhibited at a much higher pH than the pH at which the growth of rhizobia in axenic culture is hindered, it would appear that pH affects the plant much more than the bacteria during the nodulation process. However, since the plants' growth was not affected, the factor is probably a rhizobia mediated and pH sensitive.

This study failed to demostrate the activity of polygalacturonase which is in support of the work of Thomas and Elkan (52), Macmillan and Cook (30). The results are in contradiction to those of Fahraeus and Ljunggren (13), and Munns (37).

Failure to demonstrate polygalacturonase activity could have been due

to the fact that the source of enzyme used was obtained from culture solution, 20 hours after inoculation. Clover seedlings may have been too old or too young. Alternatively, some cation such as K^+ as mentioned by Thomas and Elkan (52), might have interfered with the reaction so that the enzyme was rendered inactive. If polygalacturonase had been demonstrated, then it might have been possible to focus the pH specific point. However, it could be suggested that, although increase in numbers of rhizobia on the rhizosphere is not specific, any factor such as pH which affects increase in rhizobia numbers, might indirectly affect infection thread initiation.

There is little doubt that soil pH has a marked effect on the survival and proliferation of rhizobia. Since low pH affects nodulation, liming program in legume culture is proposed for the acidic soils of East Africa. Response to liming has been obtained by Loos and Louw (27), Lowther and Lonergan (29), and Bryan (4), and others. Liming would not only ensure ideal nodulation conditions for the rhizobia (microsymbiont), but would also benefit the host (macrosymbiont), by making nutients such as phosphate, and calcium more available, reducing Mn, Fe and Al toxicity to the plant. A word of caution on liming of tropical soils, however, is necessary at this point. Tropical soils, mainly oxisols (latosols), ultisols etc. have low base status and the cations specially K^+ are in delicate balance so that liming beyond pH 5.5 is likely to upset the cation balance and nullify the benefit of liming. Nevertheless, liming may not be a panacea to the acid tropical soils because generally, the tropical species of rhizobia would nodulate in an unamended soils, whereas the temperate species only nodulate if $CaCO_3$ is added (4).

Meanwhile, as our understanding of the ecology of Rhizobium and the legume - Rhizobium association is improved subsequent observations must be interpreted with care due to changes to the biological, inorganic

and organic environment. As more work continues on the search for specificity, it should be stressed that there is need for more work on the ecology of symbiosis. Many factors are known to influence nodule formation and nodule function under ideal conditions. Much less is known about the importance and role of various field factors such as moisture, aeration, temperature and pH etc. Only by developing an understanding of those factors and the complex interactions in which rhizobia and legume are involved can we expect to achieve the full potential of the legume-rhizobia symbiosis.

SUMMARY

Three of the 5 Rhizobium trifolii strains studied consistently
d ineffective nodules on 6 Trifolium spp. The other 2 Rhizobium
olii strains were effective on T. fragiferum (strawberry white clover),
epens (Ladino white clover) and T. pratense (Pennscot and Marmoth red
ers) varieties from New York State. Strains T_1 and Rt_8 were ineffec-
on T. fragiferum, T. repens and T. subterranean (subterranean clover)
.eties from Israel and T. semipilosum (Kenya white clover), a variety
Kenya. It was concluded that effective temperate rhizobia may not
essarily be effective on sub-tropical or tropical clover varieties.

Various methods for testing nodulation and nitrogen fixation were
luated. Leonard jars and pots with sand as media gave good results
nitrogen fixation when used in the greenhouse rather than in the small
rth chambers. Shoot to root ratio, number, size and color of nodules,
weight and nitrogen content of plants were used in rating ineffec-
veness.

Studies of rhizobia in pure culture revealed that there was no cor-
lation between production of indole acetic acid from tryptophan and
fective or ineffectiveness. However, 2 of the ineffective strains con-
rted more tryptophan to indole acetic acid than the effective strains.
ffective strains were fast growers and produced slight amounts of acid
hen alkali in liquid YEM media. None of the ineffective strains grew
t pH 4.5, and none grew in nutrient broth. The effective strains were
ast growers, grew at pH 4.5, proliferated in nutrient broth, and pro-
luced alkali. Effect of extreme pH were only bacteriostatic for rhizobia
because cells exhibited catalase activity from pH 4.5 to 11.0 with best
ctivity on the alkaline side.

The growth of the rhizobia was greatly affected at pH 5.0, and these inhibition of bacterial growth coincided with poor nodulation. High amount of Ca in liquid media stimulated growth of rhizobia at pH 5.0. No stimulation was observed at pH 4.5. In liquid YEM media, high cell concentration in initial inoculum did not alleviate the effect of low pH of 4.5.

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