

AN ABSTRACT OF THE THESIS OF

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Nodulation Characteristics of *Rhizobium Trifolii*

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Nodulation success by two indigenous *Rhizobium trifolii* serogroups, 6 and 36, on subterranean clover (*Trifolium subterraneum* L.) was affected by the addition of phosphate to Abiqua soil (Ultic Haploxeroll). To understand the impact of phosphorus nutrition on the survival and nodulation characteristics of the two serogroups, studies were carried out to determine: (i) the growth characteristics of representative serogroup isolates, 6 and 36, in defined nutrient solution with phosphate concentrations typical of soil solution; and (ii) the influence of phosphate stress upon the nodulation characteristics of the two isolates at pH 5.5 and 6.5. An iron (III) oxide dialysis system was used to provide growth medium buffered at 0.00, 0.28, 0.51, 0.87, and 6.50 uM phosphate at pH 5.5. After depleting the internal P content of the organisms, the external critical phosphate requirements of 6 and 36 were found to be significantly different (0.56 and 0.93 uM

respectively). Despite the relatively small difference between the critical phosphate requirements, 6 adapted to phosphate-depleted medium far better than 36 by maintaining viability in 0.00 uM P medium, whereas 36 lost viability within 48h in medium having  $\leq$  0.56 uM P. At pH 5.5, P-deficient 36 was inferior to P-deficient 6 by taking 2 to 4d longer to initiate nodulation and forming less nodules in P-depleted plant growth medium. Nodulation by P-starved 36 was improved by either the presence of phosphate at pH 5.5, or by a higher pH of the plant growth medium depleted of phosphate.

Rhizoplane enumeration showed that both 6 and 36 could proliferate even under stringently low inorganic phosphate conditions (5.4 to 9.2 generations in 7d) and achieved about 9 generations at 21d. P-deficient 36 proliferated significantly less than luxury P grown 36 regardless of the P status of the plant growth medium. The numbers of nodules formed by 6 and 36 were not related to the rhizoplane densities of the bacteria.

Influences of Phosphate on the Growth and  
Nodulation Characteristics of  
Rhizobium Trifolii

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Influences of Phosphate on the Growth and  
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CHAPTER I

Introduction

LITERATURE REVIEW ON THE EFFECTS OF PHOSPHATE  
ON THE GROWTH AND NODULATION CHARACTERISTICS  
OF RHIZOBIUM TRIFOLII

Since the oil crisis of the mid-seventies, much effort has focused on expanding the use of nitrogen fixing legumes throughout the world. Research has been extensive in defining the major limitations to their productivity. Superior nitrogen fixing strains of Rhizobium sp. have been obtained, and screened for attributes considered necessary for success under the stresses typical of many soil conditions. Soil acidity (Keyser and Munns, 1979), temperature extremes (Munevar and Wollum II, 1982), and low phosphate levels (Cassman et al., 1981) have been prominent. In this latter context, a review on how phosphate affects Rhizobium and its symbiotic relationship with legumes is presented.

Phosphate is one of the most deficient nutrients in soils throughout the world, especially in highly weathered soils which contain a high proportion of amorphous clay minerals, Fe or Al oxides. Because of the strong adsorption of phosphate by these soils, the availability of phosphate to plants, and possibly microbes present in these soils is often limited. Therefore, the success of the legumes and their symbionts will be very much dependent on their tolerance of low phosphate or the application of phosphate

fertilizer to the soils. According to Fox (1978), the external soil solution phosphate levels required by most plants to achieve 95% of their maximum yield ranges between 0.3 to 12.9  $\mu\text{M}$  P in soil solution. For subterranean clover (Trifolium subterraneum L.), the soil solution phosphate requirement is 5.2  $\mu\text{M}$  (Ozanne and Shaw, 1967). The critical phosphate requirement for the growth of Rhizobium varies greatly between species and strains, and ranges between 0.05 and 1.00  $\mu\text{M}$  P in a phosphate buffered system (Cassman et al., 1981a). It is still not clear from phosphate response studies as to which symbiont partner is more sensitive to the phosphate level of the soil solution and if variation in rhizobia sensitivity is important in predicting growth responses of legumes to P fertilization. In this review, emphasis will be placed on how phosphate influences: (i) the overall efficiency of nitrogen fixation in legume / Rhizobium symbioses; (ii) the nodulation of legumes by their respective Rhizobium; (iii) the survival and growth of Rhizobium in soil and in defined medium; and (iv) the growth and symbiotic ability of Rhizobium through interactions of phosphate with other factors in soil.

Effect of phosphate on nitrogen fixation in  
legume/Rhizobium symbioses

The importance of phosphorus in symbiotic nitrogen

fixation was first demonstrated by Truesdell (1917). Later Trumble and Shapter (1937) also showed that total nitrogen yield of subterranean clover was increased by applying phosphate dressings. However, the increase in total nitrogen assimilated may have simply been an indication of better plant growth on soil nitrogen in response to phosphate fertilizer and may have had nothing to do with enhanced nitrogen fixation. McLachlan and Norman (1961) obtained evidence that phosphate addition would increase the amount of nitrogen fixed by subterranean clover. A negative interaction was observed between the percentage of N derived from nitrogen fertilizer and the application of phosphate, even though both the fertilizers improved the nitrogen content and growth of the plant. Besides subterranean clover, similar results were also observed with Stylosanthes humilis (Andrew and Robins, 1969a; Gates and Wilson, 1974). Different observations were made by Gates (1974) and Tyson (1955). Alleviation of phosphate deficiency had no effect on nitrogen concentration in the shoots of S. humilis and T. subterraneum but the total nitrogen assimilated from both nitrogen fixation and soil nitrogen and also plant growth yield were increased. The discrepancy between the effects of P upon  $N_2$  fixation by different legume species may have been due to the dilution effect of the plants or through sampling of plant tissue at different stages of

maturity.

#### Effect of phosphate on nodulation

In general, phosphate application can increase nodule number and weight (Munns, 1977). De Mooy and Pesek (1966) found that maximum nodulation of Glycine max (soybean) required very high levels of applied phosphate and potassium salts. Findings of other researchers also showed that increases in phosphate would significantly increase nodulation of soybean by Bradyrhizobium japonicum in pot and field conditions (Wilson, 1917; Heltz and Whiting, 1928). However, contrasting results were obtained with soybean, white and subterranean clovers. Perkins (1924) found that 22 mmoles P/kg soil sharply reduced the number of nodules of soybean. Fellers (1918) found that P improved nodulation of soybean only when sufficient Ca was applied. Hallsworth et al. (1964) found that phosphate addition had no effect on the numbers of nodules formed on subterranean and white clovers. In the latter case, a significantly greater total weight of nodules as well as a greater weight per gram of root were observed with the application of phosphate. This phenomenon was not observed for subterranean clover. In none of the studies described above were attempts made to determine if P applications influenced the nodule occupancy by inoculant or indigenous strains of Rhizobium. Such

changes, or lack thereof, might have been responsible for the variation in nodulation and effectiveness of nitrogen fixation in response to P application. Recently, Almendras and Bottomley (1985) presented preliminary evidence which showed that with the application of phosphate, the ratio of nodules occupied by the indigenous R. trifolii serogroups 6 and 36 would change even though the total numbers of nodules on the subterranean clover roots were unaffected. Therefore, it is very important to understand how different strains within an indigenous population of Rhizobium are affected by the external phosphate concentration. For example, an extreme situation might be one where ineffective strains in a soil are activated and enhanced in nodulation by phosphate application. Obviously then, the P addition to the soil would unlikely result in an improvement of nitrogen fixation.

#### Effect of phosphate on the growth of Rhizobium

Survival in soil is very important for Rhizobium because this will ensure that the bacteria persist in sufficient numbers to promote nodulation of the host species upon its return to the soil (Parker et al., 1977). It is still unclear how the outcome of nodulation of the host plant is related to the magnitude of the soil population of Rhizobium. Moawad et al. (1984) reported that no relationship was found between

the size of the soil population of B. japonicum serogroup 123 and its nodulating ability. Amarger and Lobreau (1982) suggested, however, that the population of a particular strain of Rhizobium present in soil was correlated with the number of nodules that it could initiate. Therefore, any factors that can increase the population of a strain of Rhizobium in a soil, or result in enhanced physiological activity will probably be beneficial to the nodulation ability of that strain. Truesdell (1917) found that phosphate caused large increases in the growth and activity of R. meliloti. Walker and Brown (1935) also observed that populations of both R. trifolii and R. meliloti in soil were increased by phosphate addition. Recently, Cassman et al. (1981a) found that different strains of B. japonicum had different tolerances to low phosphate concentrations and they suggested that those with less efficiency at sequestering P at low levels might have difficulty colonizing phosphate deficient soils or plant rhizospheres. Cassman et al. (1981b) also showed that the extent to which cells could store and then use P to support subsequent growth varied with the strain. Therefore, those strains that could store more P during a phosphate sufficient period might have a competitive advantage for survival, and for colonization of nodulation sites in rhizospheres when soils were phosphate depleted. Smart et al. (1984a) showed that

the growth of R. trifolii, strain WU95, and two strains of cowpea Rhizobium was controlled by the attainment of a critical internal P level. In addition, their data suggested that strains of slow growing cowpea Rhizobium did not accumulate polyphosphate under phosphate sufficient conditions nor derepress alkaline phosphatase in low P medium in contrast to two strains of fast-growing rhizobia. Beck and Munns (1984) screened 40 strains from six species of Rhizobium in low phosphate defined medium and concluded that the ability to store P at high levels was less important than the uptake of P at low levels and its subsequent efficiency of utilization.

Effects of other soil factors on legume / Rhizobium symbioses through their interactions with phosphate

In soil, other factors can also interact with phosphate directly or indirectly to affect the successfullness of nodulation. For examples, changes of pH,  $\text{Ca}^{2+}$ ,  $\text{Al}^{3+}$ , or  $\text{Mn}^{2+}$  in soils can vary the availability of phosphate to legumes and, or, Rhizobium. The influences of these factors will be through the precipitation of phosphate in soil or through influence on the membrane permeabilities of the symbionts.

Phosphate deficiency is common in acid soils and there is evidence that liming can partially overcome the deficiency (Andrew, 1978; Rhue and Hensels, 1983;

Haynes, 1984; Holford, 1985). Several mechanisms are possible with increases in either pH or  $\text{Ca}^{2+}$ , or both being implicated in improving the availability of P to the symbionts. Hendrix (1967) found that bean plants absorbed phosphate much faster at lower pH. On the other hand, reduction of soil pH reduces the availability of phosphate to plants (Kawai, 1980). Keyser and Munns (1979) found that increasing the pH of defined growth medium could increase the tolerance of Rhizobium towards low P concentrations. They also observed that the pH-phosphate interaction was strain dependent.  $\text{Ca}^{2+}$  can also interact with the uptake of phosphate by legumes. Robson et al. (1970) suggested that  $\text{Ca}^{2+}$  would bind to the negatively charged membranes of the root cells of annual legumes, and, by the partial neutralization of the membrane charge, would facilitate the uptake of phosphate at low concentrations. Beck and Munns (1985) showed that R. meliloti required at least 3 mM  $\text{Ca}^{2+}$  in a defined growth medium in order for it to take up P or even to utilize its storage P under low P conditions.  $\text{Ca}^{2+}$  has also been proven to facilitate the uptake of P in other microorganisms (Rigby et al., 1980; Poindexter, 1983).

Aluminum can interact with phosphate either directly in the soil or through the symbionts. At low pH, hydrated  $\text{Al}^{3+}$  will precipitate with phosphate and diminish the availability of P in the soil. Increases of

$\text{Al}^{3+}$  concentration inside plant tissue impede transport of phosphate resulting in an increase in the P content in roots but lower P concentration in plant tops (Foy and Brown, 1964; Munns 1965a, b). Andrew and Vanden Berg (1973) found that P uptake by aluminum-treated M. sativa roots was enhanced initially but later P was desorbed back into the surrounding medium. Rhizobium has been shown to be very sensitive to Al toxicity. Keyser and Munns (1979) showed that 26 out of 65 strains of slow-growing rhizobia virtually stopped growing in defined growth medium containing 50  $\mu\text{M}$   $\text{Al}^{3+}$ . They also observed that an increase of either phosphate or pH would partially overcome the  $\text{Al}^{3+}$  toxicity. Thornton and Davey (1983) and Wood and Cooper (1984, 1985) showed that phosphate addition could increase the  $\text{Al}^{3+}$  tolerance of R. trifolii. Nodulation of T. repens was found to be inhibited by 50  $\mu\text{M}$   $\text{Al}^{3+}$  in 10  $\mu\text{M}$  P growth medium at pH < 5.0 but the toxic effect of  $\text{Al}^{3+}$  was overcome by either increasing the P concentration to 50  $\mu\text{M}$  or the pH to 6.0 (Wood et al., 1984).

Excess of  $\text{Mn}^{2+}$  in soil can also reduce plant growth, nodule numbers and weight (Andrew, 1978). On agar medium, Masterson (1968) indicated that the toxic levels of  $\text{Mn}^{2+}$  to several strains of R. trifolii ranged between 15-27 mM. Wilson and Reisenauer (1970) found that growth of R. meliloti was not reduced in defined medium by 100  $\mu\text{M}$   $\text{Mn}^{2+}$ . Unlike aluminum, excess  $\text{Mn}^{2+}$

does not adversely affect the uptake of phosphate into plants (Andrew and Hegaty, 1969). Recently, Holford (1985) found that liming would make soil P more available to white clover growing on acidic, manganiferous phosphate-deficient soils. However, too much Mn<sup>2+</sup> will reduce the available P in soils by precipitation. Until now, Mn<sup>2+</sup> effects on Rhizobium growth under extremely low P are still unclear.

Occurrence of endotrophic mycorrhizae in the roots of several tropical legumes has proven to be valuable in low phosphate soils (Possingham et al., 1971; Mosse et al., 1973). It was also shown that the mycorrhizae association could relieve the phosphate stress on the nodulation of some legumes (Crush, 1974). Later, Abbott and Robson (1977) found that inoculation of subterranean clover with mycorrhizae could stimulate nodulation, phosphate uptake, and plant growth in phosphate limited soil.

#### Thesis objectives

Despite all the research interest and controversy surrounding phosphate effects on legumes, no comprehensive studies to date have been reported where phosphate has been shown to influence the nodulating success by different members of soil-borne rhizobia communities and where the effect was related to variations in phosphate sequestering ability of those

strains. Recently, Almendras and Bottomley (1985) found that application of phosphate to Abiqua soil would influence the nodule occupancy by the indigenous R. trifolii serogroup 36 on subterranean clover cv. 'Mt. Barker'. They showed that with the addition of P, percentage nodule occupancy of 36 increased from 18% to almost 50% but 6 remained unchanged between 50 to 85%. Therefore, the nodulation success of 6 and 36 might be related to differences in their ability to sequester phosphate from the nonfertilized soil or the abilities of the bacteria to colonize the rhizospheres of the host plants and activate the nodulation genes at the low P concentrations typical of plant rhizospheres. The objectives of this thesis are to determine: (i) the growth characteristics of representatives of indigenous serogroups 6 and 36 under different phosphate concentrations typical of soil solution; and (ii) the nodulation characteristics of 6 and 36 on subterranean clover cv. 'Mt. Barker' under low phosphate conditions.

## CHAPTER II

Growth characteristics of Rhizobium trifolii  
under low phosphate conditions

## ABSTRACT

The percentage nodule occupancy by indigenous R. trifolii serogroup 36 on subterranean clover can be stimulated by amending the Abiqua soil with phosphate and that occupancy by serogroup 6 is not affected. The present study was conducted to determine the critical phosphate concentrations required by 6 and 36 to commence proliferation and their growth characteristics under different phosphate concentrations. To prevent the interference of storage phosphate on the growth of the bacteria under low phosphate conditions, the bacteria were phosphate starved prior to inoculation into growth medium containing different phosphate concentrations. Growth medium was adjusted to zero (contaminating phosphate in the growth medium was removed by iron (III) oxide powder), 0.28, 0.51, 0.87, and 6.50 uM phosphate using a phosphate-charged iron (III) oxide dialysis system. Phosphate-deficient cells of 6 and 36 showed different growth responses to the different phosphate concentrations. Under zero phosphate conditions, the majority of cells of 6 remained viable throughout the first 78h of incubation. In the case of 36, no viable cells were detected after 27h. Strain 36 commenced proliferation in the presence of 0.93 uM phosphate, whereas 6 required a concentration of 0.56 uM phosphate. At supracritical concentrations

of phosphate, strain 6 recovered more rapidly from phosphate stress than 36. Increasing  $\text{Ca}^{2+}$  concentration from 1.2 to 3.3 mM decreased the recovery time required for P-starved 36 to grow at its critical P level (0.90  $\mu\text{M}$ ), but had no effect in subcritical P concentrations.

## INTRODUCTION

Phosphorus is an essential element required for the growth of Rhizobium and it is also one of the most deficient nutrients in agriculture soils especially those containing high proportions of allophane, Fe or Al oxides. Since solution phosphate concentrations of most soils are less than 1 uM (Reisenauer, 1966), Rhizobium strains having low tolerance to phosphate deficient environments may have difficulties in competing for nodulation with other indigenous rhizobia in the soils. In growth medium, Cassman et al. (1981a) found that several strains of B. japonicum differed markedly in their external phosphate requirement for growth. Smart et al. (1984a) found that different Rhizobium species required different levels of critical internal phosphorus to survive in phosphate deficient medium. Beck and Munns (1984) found that Rhizobium strains also differed in phosphate uptake efficiency under low external phosphate condition and concluded this might affect bacterial growth in phosphate deficient soils. Field experiments utilizing five strains of B. japonicum showed different responses to external phosphate for nodulation and nitrogen fixation (Singleton et al., 1985). All these reports indicate Rhizobium strains differ in response to external phosphate concentration but very little information has been forthcoming from

the literature to suggest that such differences do exist within, and indeed influence relative nodulating capabilities by mixtures of indigenous soil rhizobia. Recently, Almendras and Bottomley (1985) found that amending the acidic ( $\text{pH } 5.2 \pm 0.2$ ), low extractable phosphate [ $1\text{-}5 \text{ mg P (kg soil)}^{-1}$ ], Abiqua soil with monobasic phosphate enhanced the nodule occupancy by indigenous serogroup 36 of R. trifolii on subterranean clover cv. 'Mt. Barker' and had no effect on the % occupancy of serogroup 6. In this study, experiments were carried out to determine the growth characteristics of representatives of serogroups 6 and 36 when exposed to phosphate concentrations typical of soil solution using a phosphate buffered iron (III) oxide dialysis system.

## MATERIALS AND METHODS

Establishing sorption and desorption isotherms of various oxides. Iron (III) oxide powder, limonite, and goethite needle ore were obtained from J. T. Baker Chemical Co., Wards Natural Science Establishment, Inc., Rochester, N. Y., and Minerals Unlimited, Ridgecrest, Cal. respectively. Goethite was pulverized and ground to a fine power before use. Sorption isotherms were determined at 25°C as described in detail elsewhere (Fox and Kamprath, 1970). In brief, triplicate 3g samples of oxide were equilibrated with 30 ml portions of 10 mM  $\text{CaCl}_2$  containing either 24.3, 48.3, 72.6, or 96.9 umoles  $\text{KH}_2\text{PO}_4$ . The samples were shaken for one hour daily over a 14 day period, and the phosphate (P) remaining in the supernatants was determined by a modification of the method of Murphy and Riley (1962) described by Throneberry (1974). Phosphate desorption characteristics were obtained by shaking intermittently ( $1\text{h d}^{-1}$ ) the air dried samples from the adsorption experiment with 30 ml portions of 10 mM  $\text{CaCl}_2$  at 25°C for 6 days and phosphate concentrations in solution analyzed.

Phosphate analysis. In order to gain a greater color intensity, the proportions of sample and reagent were adjusted to 2.5 ml of sample, 2 ml distilled water and 1.5 ml of color developing reagent. The absorbance of

the blue color complex was measured in a 1 cm path length cuvette at 890 nm.

Strains of Rhizobium trifolii. The bacteria used in these studies were the parent isolates of two indigenous serogroups 6 and 36 found in Abiqua soil and described in detail elsewhere (Dughri and Bottomley, 1983).

Growth of 6 and 36 in phosphate-supplemented (+PGG) and phosphate-depleted (-PGG) media. Bacteria were grown in a previously described defined medium (Dughri and Bottomley, 1983) modified as follows. Glucose (10g) was substituted for mannitol and autoclaved separately; chelated Fe was supplied by adding  $\text{Na}_2\text{EDTA}$  (27 uM) and  $\text{FeCl}_3$  (14 uM) and the vitamin mixture of Chakrabarti et al. (1981) was used. 10 mM MES (2[N-morpholino]ethane sulphonic acid) was used as a buffering agent and the medium was adjusted to pH 5.5 with HCl.  $\text{K}_2\text{SO}_4$  (0.25 mM) was added to P-depleted glucose-glutamate medium (-PGG) to substitute for the omission of the  $\text{K}^+$  component of  $\text{K}_2\text{HPO}_4$  and pH was adjusted to 5.5 with KOH.

Cultures were grown in 30 ml of medium contained in cotton-stoppered pyrex test tubes (20x2.5cm) at 30°C in a thermostatted water bath and bubbled continuously with filter-sterilized air at a rate of about 10 ml per min. To produce P-deficient cells, the following procedure was adopted. One ml of an early stationary phase culture grown under phosphate-supplemented (2.85 mM P) conditions (+PGG) was used to inoculate sterile

-PGG. Growth was monitored until the culture reached a constant turbidity (stationary phase). One ml of this culture was backtransferred again to -PGG and growth monitored until the culture reached constant turbidity.

Determination of the dry weight, P content, and morphology of cells of 6 and 36 grown under different P regimes. 6 and 36 were grown in +PGG and then P-starved through two growth cycles as described above. The cell densities of the cultures of 6 and 36 in the second P-starved cycle were measured at their stationary phases by plate counting on yeast extract mannitol agar (YMA) (Vincent, 1970). The bacteria were harvested by centrifugation at 25,000 g for 15 min, the cell pellets were washed twice with distilled water, dried at 55°C, and the dry weights of 6 and 36 were determined. Cells of 6 and 36 grown under P-supplemented and one or two cycles of P deficiency were analyzed for their total P content by either perchloric acid digestion with MgCl<sub>2</sub> (Brookes and Powlson, 1981) or by concentrated acid and ammonium persulphate digestion (USEPA, 1974). P contents of the digests were determined as described above. Smears of cells of 6 and 36 grown under the different P conditions were prepared and the cellular dimensions (length and width) were determined by immunofluorescence microscopy after staining with specific fluorescent antibodies as described elsewhere (Demezas and Bottomley, 1986a).

Growth of P-starved 6 and 36 at different P concentrations. Samples of iron (III) oxide powder unloaded and also preloaded with 8, 13, 16, 32 umoles P per gram oxide were used to establish a range of P concentrations in -PGG of 0 to 6.54 uM. Three gram samples of prepared oxide and 4 ml distilled water were placed into cellulose dialysis tubing (16 mm diameter Spectrapor<sup>TM</sup> dialysis membrane with average pore radius permeability of 24 Å) which was sealed at one end. After all gas bubbles had been carefully removed from the tubing, the other end was also sealed. The iron (III) oxide-containing sacs were placed separately into acid washed pyrex test tubes containing 40 ml of -PGG. Samples were then autoclaved for 30 min and incubated at 30°C with continuous aeration for 6 days prior to inoculation. Solution P concentrations of the media were analyzed prior to inoculation to verify establishment of a constant P concentration. Cultures of 6 and 36, P-starved as described above, were diluted with sterilized 0.85% NaCl solution (w/v) to about  $10^5$  cells  $\text{ml}^{-1}$  and used to inoculate the glucose-glutamate (GG) medium containing different amounts of P, to give an initial cell density of approximately  $10^3$  cells  $\text{ml}^{-1}$ . Growth was followed by determining viable counts on YMA plates. Phosphate concentrations of the media were also determined at 24 and 72h after inoculation (data not shown). Attachment of 6 and 36 to the dialysis tubing

was also assessed at different time intervals by shaking the dialysis sac in 50 ml of partially hydrolysed gelatin in ammonium phosphate (Kingsley and Bohlool, 1981) for 15 min, and plate counting the extract on YMA plates.

Ca<sup>2+</sup> effect on the growth of 36. Samples of -PGG containing calcium ion concentrations of 0.5, 1.5 and 2.5 mM were prepared and equilibrated at 30°C with iron (III) oxide preloaded with 16 umoles P (g oxide)<sup>-1</sup>.

Ca<sup>2+</sup> was determined by atomic absorption spectroscopy, and P determined as described above, before inoculation with bacteria. After inoculation, growth was followed by plate counting on YMA at time intervals.

Mn<sup>2+</sup> effect on the growth of 6 and 36. The effect of Mn<sup>2+</sup> on the growth of P-starved 6 and 36 was carried out in low (1.50 uM) and high (2.85 mM) P-containing growth medium. Portions of a 0.4 um filtered sterilized MnCl<sub>2</sub> solution were added to media to produce final Mn<sup>2+</sup> concentrations of 0.5, 50, 100, 150, 200 and 1,000 uM. P-starved cells of 6 and 36 were inoculated into these media to provide an initial optical density of about 0.01 (2.5 cm diameter pyrex test tube). The bacterial cultures were incubated as described above and growth was followed turbidimetrically at 660 nm. The exponential phase generation times of the bacteria at the different Mn<sup>2+</sup> concentrations were determined.

## RESULTS

The phosphate sorption isotherms of synthetic iron (III) oxide, limonite and goethite were established to compare their phosphate buffering capacities (Fig. 1). Besides having the highest sorption ability, the synthetic iron (III) oxide powder produced the steepest slope of the three tested iron oxides, implying a greater phosphate buffering capacity. In the case of desorption isotherms, both the limonite and synthetic iron (III) oxide curves showed hysteresis effects and the latter consistently required a higher reserve P content than the former to produce the same solution P concentration. This latter characteristic, along with the reproducibility from different batches of the synthetic oxide and the cost factor determined the decision for choosing synthetic iron (III) oxide powder as the buffering agent of our system.

The generation times of 6 and 36 were rather similar (3.4 and 3.5h respectively) in P-supplemented defined bacterial growth medium at pH 5.5 and 30°C (Fig. 2). The generation times of both 6 and 36 during the exponential phase of the first growth cycle in -PGG were similar, yet longer (5.0 and 4.6h respectively). The terminal cell density (approximately  $3 \times 10^8$  cells  $\text{ml}^{-1}$ ) was significantly lower than the final cell density achieved in P-supplemented medium for both 6 and 36.

During the second P-depleted growth cycle, the generation time of 36 was extended to 9.8h while the generation time of 6 remained unchanged (5h). Despite the difference, the terminal cell densities of the second phosphate-starved cultures were similar, yet substantially lower than the values achieved from the first P-depleted growth cycle.

Despite the different growth rates under P-deficient conditions, the internal P content of 6 and 36 was similar regardless of the P status of the growth medium (Table 1). Differences between the two strains were also not apparent when P content was related on a single cell basis (Table 2). Under P-depleted conditions, both 6 and 36 changed morphologically in a similar manner with their weight per cell being similar, and lengths increasing to twice that of cells grown under P-sufficient conditions (Table 2). P-starved 6 was observed on occasion to express a pleiomorphic morphology.

P-depleted 6 and 36 showed different growth responses when exposed to different phosphate concentrations (Fig. 3 and 4). In the case of 6, the growth patterns were of three types (Fig. 3a, b, c). When solution concentrations of phosphate were < 0.28 uM, cell proliferation did not occur, yet the inoculum remained viable for at least 72h. At concentrations between 0.45 to 0.56 uM P and between 0.81 to 0.93 uM P,

6 showed ability to grow after an extended lag phase of 24h. With solution phosphate concentrations  $\geq 6.45 \mu\text{M}$ , P-starved 6 commenced proliferation without any lag period, and the growth rate was equivalent to that achieved in normal phosphate-supplemented (2.85 mM P) growth medium. The behavior of P-starved 36 was quite different under the same circumstances (Fig. 4). At P concentrations  $< 0.56 \mu\text{M}$ , 36 lost viability within 48h and commenced proliferation only at phosphate concentrations of 0.81 to 0.93  $\mu\text{M}$  after an extended 48h lag phase. The lag phase of 36 decreased as it was exposed to greater external phosphate concentrations. Differential attachment of 6 and 36 to the dialysis tube was found not to be a factor affecting the number of viable 36 recovered from in the growth medium (data not shown).

The calcium and manganese concentrations of the growth medium were influenced by exposure to the iron (III) oxide in its uncharged or charged states (Table 3). Calcium concentrations were greater when the iron (III) oxide was loaded with phosphate whereas manganese concentration of the nutrient solution was greatest after exposure to uncharged iron (III) oxide and decreased with increasing phosphate levels.

Both the growth rate of 36, and the time to recover from P starvation, were improved in 0.90  $\mu\text{M}$  P growth medium as the  $\text{Ca}^{2+}$  concentration increased from

1.20 to 2.20 or 3.34 mM (Fig. 5). However, when the phosphate concentration was lowered to a subcritical level, 0.50 uM, the  $\text{Ca}^{2+}$  effect disappeared (data not shown).

Both P-starved 6 and 36 showed no adverse growth effects to solution  $\text{Mn}^{2+}$  levels ranging from 0 to 1,000 uM in either luxury (2.85 mM) or low (1.23 to 1.80 uM) P conditions (Table 4). The average generation times of 6 and 36 were 8.8 and 7.9h respectively under low P conditions and were about double those under luxury P conditions. No significant differences in the lag phase of either organism were found in any treatment.

Figure 1. Sorption and desorption isotherms of different iron oxides at pH 5.2. Solid and broken lines represent sorption and desorption isotherms respectively of: synthetic iron (III) oxide, ● ; limonite, ○ ; and goethite, ■ .

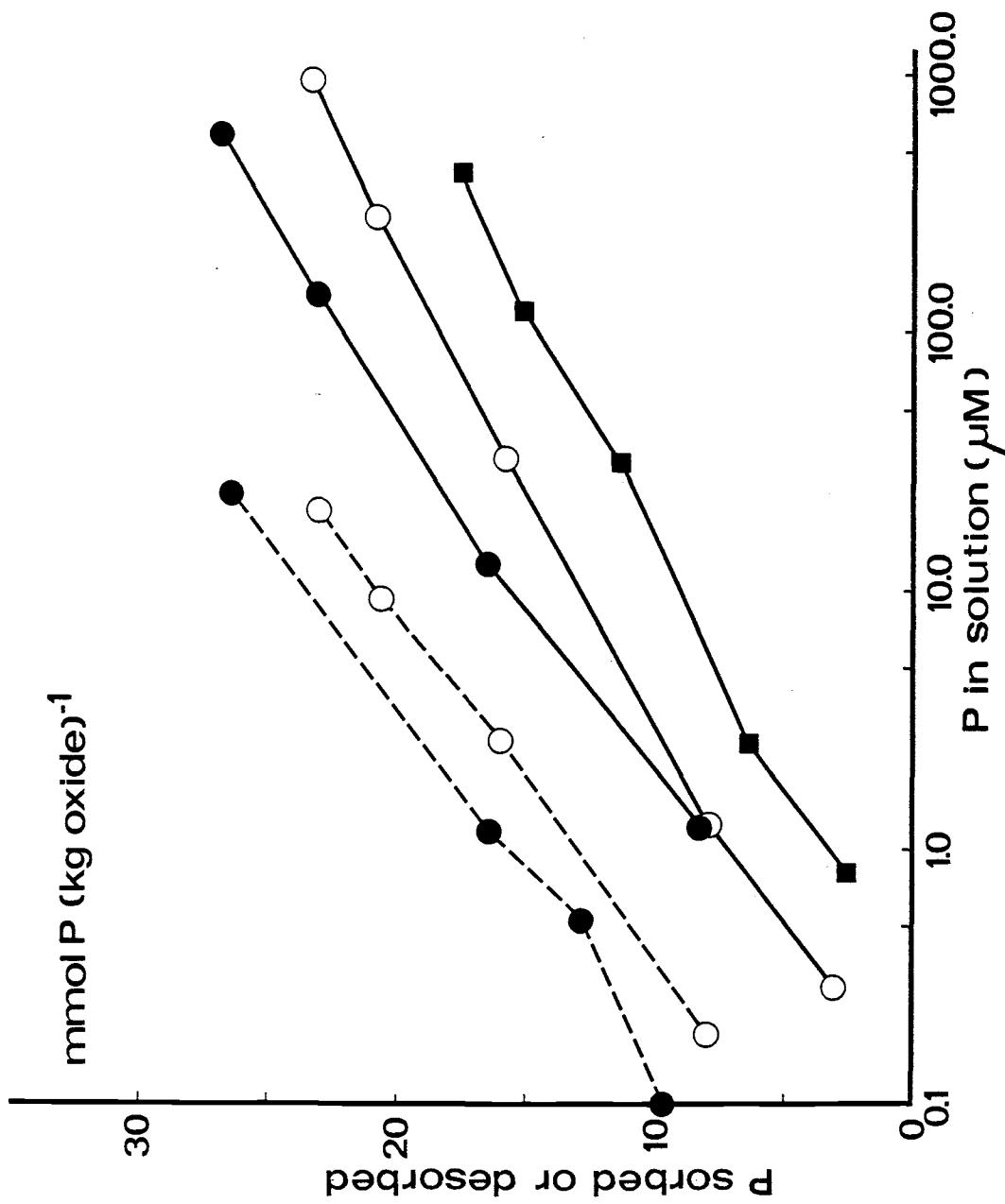


Figure 1

Figure 2. Growths of R. trifolii strains 6 and 36 under P-rich and P-depleted conditions. 2.85 mM P, ●; bacteria transferred from 2.85 mM P to -PGG, ■; second P-starvation cycle in -PGG, □. Points on each curve represent the mean of 3 separate determinations. All values for standard deviations (95% confidence level) are less than the size of the symbols.

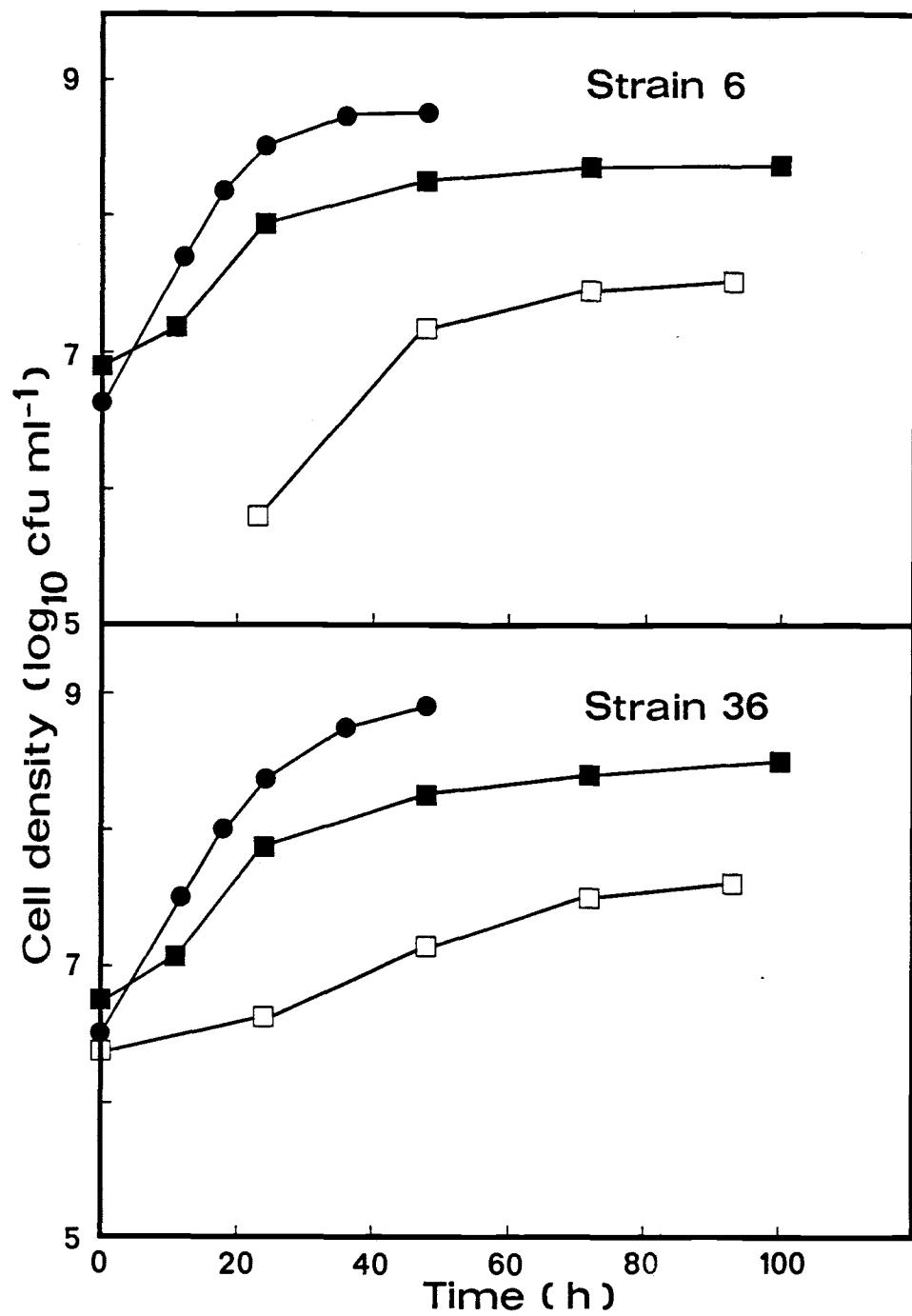


Figure 2

Figure 3. Growth of R. trifolii strain 6 in different phosphate concentrations at pH 5.5. a. 6.45-6.54 uM P, □ ; 2.85 mM P, ■ ; b. 0.45-0.56 uM P, ○ ; 0.81-0.93 uM P, ● ; c. zero P, ◉ ; < 0.28 uM P, ★ . Vertical bars represent standard deviations (SD) at the 95% confidence level. No vertical bar indicates SD is smaller than the symbol.

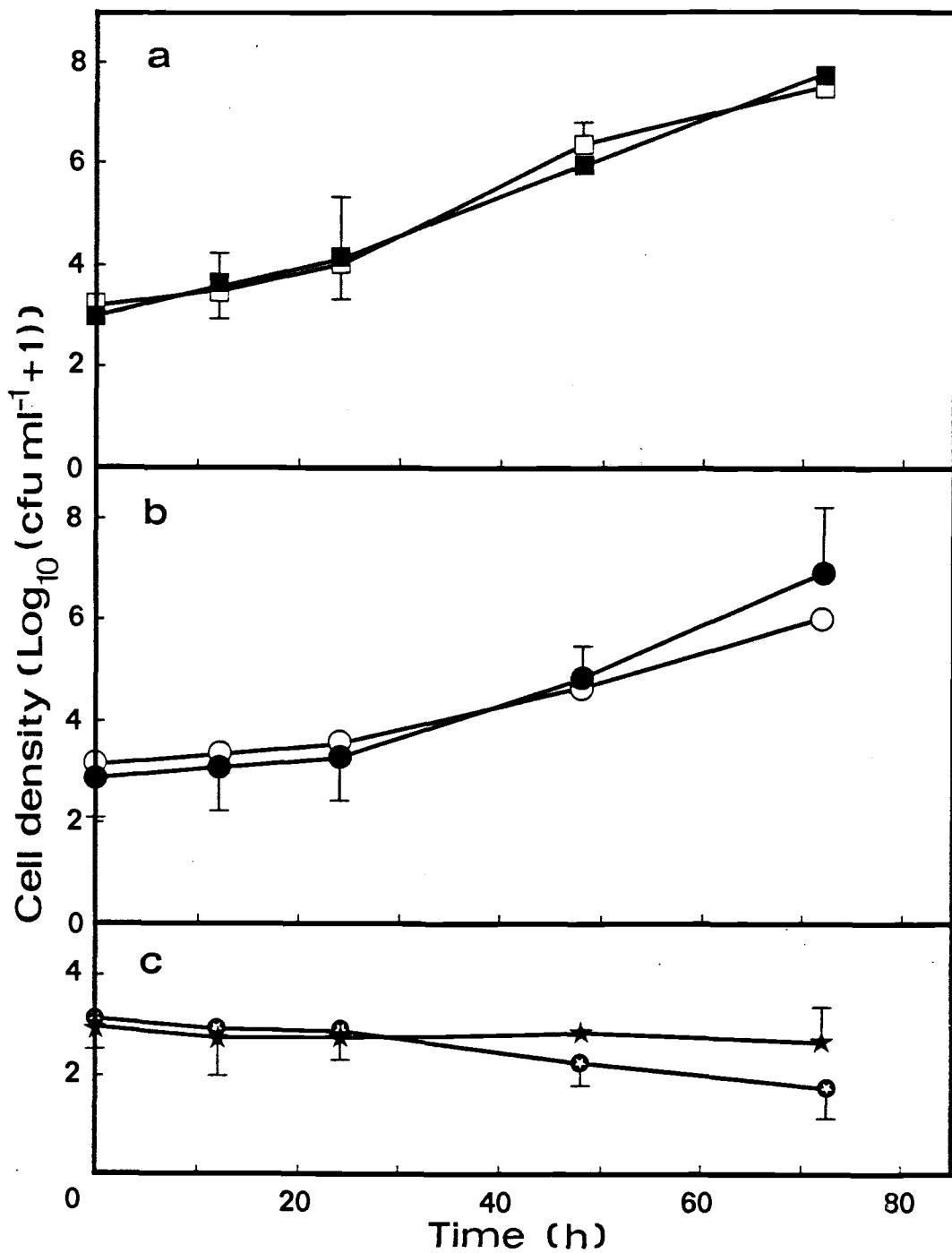


Figure 3

Figure 4. Growth of R. trifolii strain 36 in different phosphate concentrations at pH 5.5. a. zero P,  $\star$ ;  $< 0.28 \mu\text{M}$  P,  $\blackstar$ ;  $6.45-6.54 \mu\text{M}$  P,  $\square$ ;  $2.85 \text{ mM}$  P,  $\blacksquare$ ; b.  $0.45-0.56 \mu\text{M}$  P,  $\circ$ ;  $0.81-0.93 \mu\text{M}$  P,  $\bullet$ . Vertical bars represent standard deviations (SD) at the 95% confidence level. No vertical bar indicates SD is smaller than the symbol.

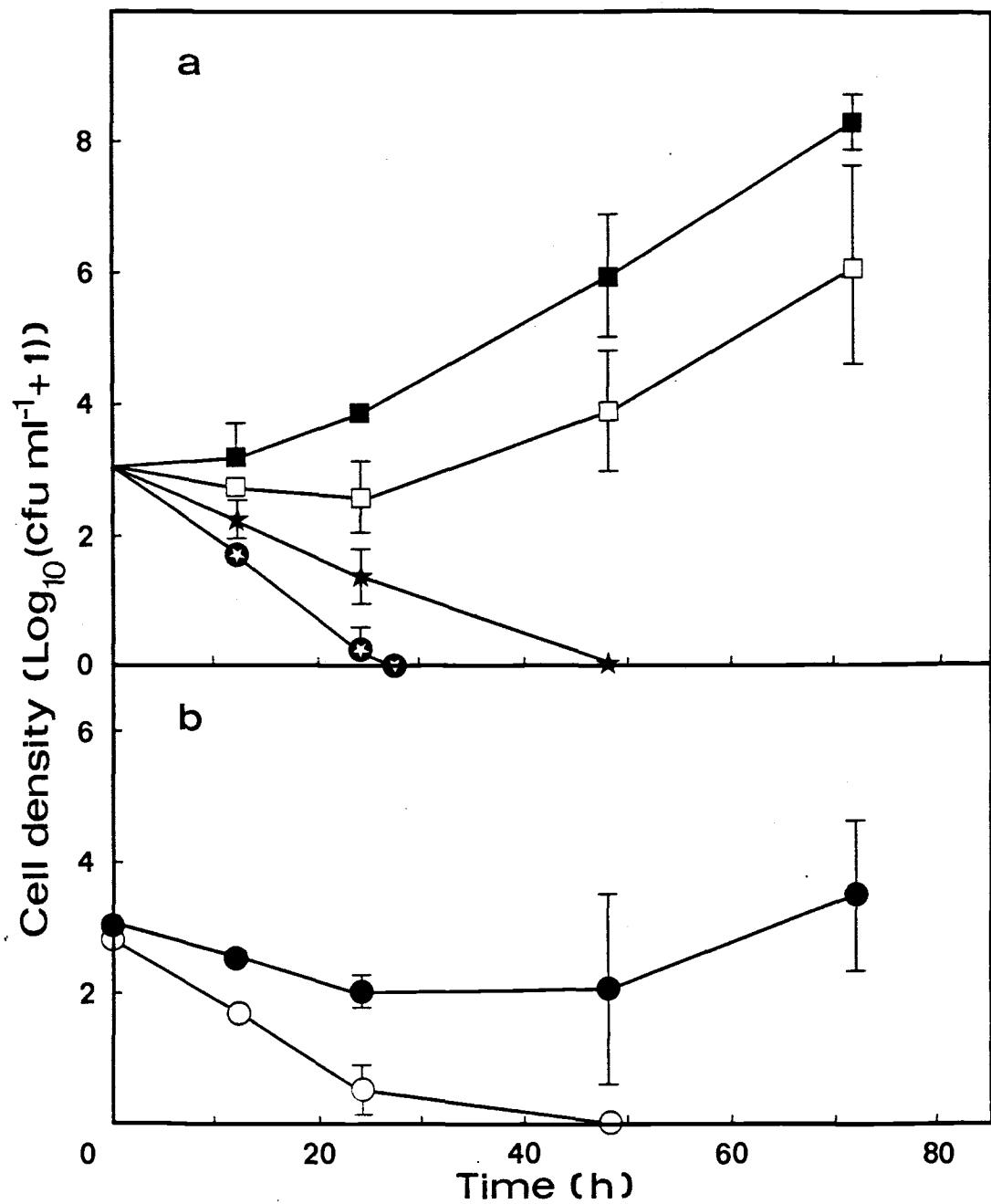


Figure 4

Figure 5.  $\text{Ca}^{2+}$  effect on the growth of 36 in 0.9  $\mu\text{M}$  P growth medium at pH 5.5. 1.20 mM  $\text{Ca}^{2+}$ , ○; 2.20 mM  $\text{Ca}^{2+}$ , ■; 3.34 mM  $\text{Ca}^{2+}$ , ●. Vertical bars represent standard deviations (SD) at the 95% confidence level. No vertical bar indicates SD is less than the size of the symbol.

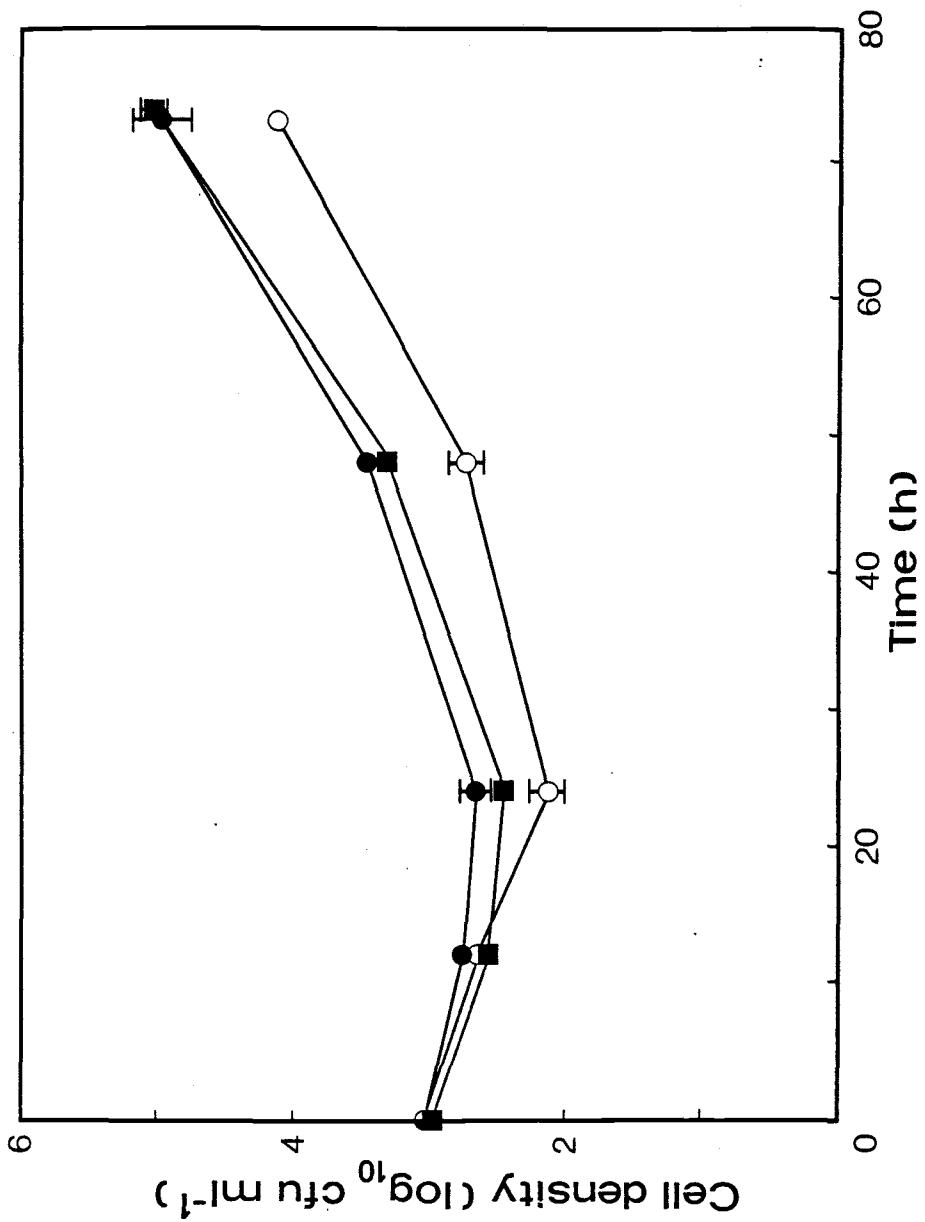


Figure 5

Table 1. Total phosphorus content of *R. trifolii* strains 6 and 36 grown under P-sufficient and P-depleted conditions.

Bacteria	P content <sup>†</sup>		
	<u>P-sufficient medium<sup>#</sup></u>	<u>P-depleted medium<sup>§</sup></u>	No. of growth cycles
		1 <sup>¶</sup>	
		mg P (g dry wt) <sup>-1</sup>	
6	7.7 <sup>a</sup> <sup>#</sup>	1.5 <sup>b</sup>	1.3 <sup>b</sup>
36	6.9 <sup>a</sup>	1.5 <sup>b</sup>	1.7 <sup>b</sup>

<sup>†</sup>P expressed on a dry weight basis and is the mean of 3 separate determinations.

<sup>#</sup>Defined growth medium supplemented with 2.85 mM P.

<sup>§</sup>Growth medium without P added (contamination level - 0.58 uM P).

<sup>††</sup>Cells transferred from P-sufficient to P-depleted growth medium were transferred to another batch of P-depleted medium and grown until constant cell density was reached.

<sup>¶</sup>Means followed by different letters are significantly different at P=0.05.

Table 2. Characteristics of R. trifolii strains 6 and 36 after growth under P-sufficient and P-depleted conditions.

Strains	Cell dimensions							
	Dry weight		P content		Length		Diameter	
	B <sub>1</sub> <sup>†</sup>	B <sub>0</sub>	B <sub>1</sub>	B <sub>0</sub>	B <sub>1</sub>	B <sub>0</sub>	B <sub>1</sub>	B <sub>0</sub>
$\cdot 10^{-13}$ g cell <sup>-1</sup> - $\cdot 10^{-15}$ g cell <sup>-1</sup> .								um
6	5.6 <sup>a</sup> <sup>§</sup>	11.0 <sup>a</sup>	4.3 <sup>c</sup>	1.4 <sup>c</sup>	3.0 <sup>e</sup>	5.2 <sup>e</sup>	1.0	1.0
36	4.6 <sup>b</sup>	8.0 <sup>b</sup>	3.2 <sup>d</sup>	1.4 <sup>d</sup>	2.8 <sup>f</sup>	5.5 <sup>f</sup>	1.0	1.0
Significance	NS	NS	NS	NS	NS	NS	NS	NS

<sup>†</sup> See material and methods for details of experimental procedures.

<sup>‡</sup> B<sub>1</sub> and B<sub>0</sub> represent cells after growth under P-sufficient and after two cycles of growth under P-depleted conditions respectively.

<sup>§</sup> Pairs of values followed by the same letter are significantly different (P=0.05) as determined by t-test comparisons.

NS = not significantly different at P=0.05.

Table 3. Influence of phosphate charged and noncharged synthetic iron oxide on the concentration of some mineral components of the bacterial growth medium.<sup>†</sup>

P concentration in growth medium treated with synthetic iron oxide <sup>‡</sup>	Inorganic S	Ca	Fe	Mn	Zn
uM	mM			uM	
0.00	5.4	0.9	10.7	118.3	4.6
0.50	5.1	1.3	12.5	55.9	4.6
6.50	5.5	1.3	12.5	14.4	3.1
Control <sup>§</sup>	5.6	0.6	16.1	5.6	1.5

<sup>†</sup>Analyses were carried out by the Plant Analysis Laboratory, Department of Horticulture, OSU, using a Jarrell-Ash inductively coupled argon plasma spectrometer.

<sup>‡</sup>Portions of growth medium were treated with synthetic iron oxide charged with zero, 12.9, and 23.2 mmol P (Kg oxide)<sup>-1</sup>.

<sup>§</sup>Growth medium without added phosphate and not exposed to synthetic iron oxide powder.

Table 4.  $Mn^{2+}$  effect on the growth rate of R. trifolii strains 6 and 36.

$Mn^{2+}$ concentration in the growth medium	Strains <sup>†</sup>			
	6		36	
	Low P <sup>‡</sup>	High P <sup>§</sup>	Low P	High P
uM				
0	9.8	4.4	7.7	5.6
5	6.7	4.4	8.4	4.8
50	8.8	4.2	7.1	5.0
100	8.6	4.1	7.1	5.2
150	9.3	4.7	8.5	4.2
200	9.5	4.4	8.5	4.8
1000	8.8	4.1	8.1	4.8

<sup>†</sup>Bacteria utilized in the inoculants had been grown under phosphate deficient conditions as described in materials and methods.

<sup>‡</sup>Low P represents 1.23 to 1.80 uM P in the growth medium.

<sup>§</sup>High P represents 2.85 mM P in the growth medium.

<sup>¶</sup>Generation time calculated from the exponential phase of growth.

## DISCUSSION

There is limited information in the literature on phosphate metabolism of Rhizobium. Surveys have shown that representatives of a variety of Rhizobium species could assimilate inorganic phosphate at very low solution phosphate concentrations similar to those encountered in soil solution (Cassman et al., 1981a; Smart et al., 1984a). Strains within Rhizobium species showed little variation in assimilating exogenous inorganic phosphate under phosphate limited conditions with the exception of one strain of slow-growing B. japonicum, USDA 142, and one fast-growing Leucaena Rhizobium strain, TAL 582 (Beck and Munns, 1984). In general, Rhizobium growth under low phosphate conditions was dependent both on the amount of storage P (Cassman et al., 1981b) and the efficiency of using the storage and external P available (Beck and Munns, 1984). In our studies, two indigenous strains of R. trifolii, 6 and 36, were shown to exhibit different growth responses under P-limited conditions despite achieving similar cell densities and cellular P contents in either P-sufficient or P-depleted medium.

P-starved 6 and 36 also showed different growth responses to medium containing different amounts of inorganic phosphate. Although strain 6 had a lower (0.56 uM) critical external phosphate requirement than

36 (0.93 uM), both values are much greater than those determined for other Rhizobium studied by Cassman et al. (1981a, b) and Beck and Munns (1984). The loss of viability by 36 in growth medium containing phosphate < 0.56 uM is extremely interesting since both of these organisms (6 and 36) were originally obtained from a soil with high P fixing capacity and in which soil solution inorganic phosphate is extremely low (< 0.5 uM) (data not shown).

Although we consider the synthetic iron (III) oxide powder to be an adequate substitute for the limonite used by others (Cassman et al., 1981a, b; Beck and Munns, 1984), two artifacts of the system were discovered and needed to be evaluated in light of the loss of viability by 36.

Beck and Munns (1985) illustrated that several strains of R. meliloti lose their viabilities in low phosphate medium containing less than 3 mM  $\text{Ca}^{2+}$ .  $\text{Ca}^{2+}$  has also been shown to improve phosphate uptake by other micro-organisms such as Caulobacter (Poindexter, 1984) and cyanobacteria (Rigby, 1980). In our case, P-stressed 36 showed improvement in recovery of growth as a result of increased  $\text{Ca}^{2+}$  only under supracritical P conditions. Since  $\text{Ca}^{2+}$  did not improve the growth or survivability of 36 under extremely low P conditions, the 0.7 mM  $\text{Ca}^{2+}$  contributed to the nutrient solution as a result of exposure to phosphate-charged iron (III)

oxide should not interfere with the interpretation of the bacterial growth at low P concentrations. However, other researchers interested in this system for controlling solution P concentrations should be aware of this 'extra'  $\text{Ca}^{2+}$  not reported previously.

$\text{Mn}^{2+}$  was also released from the synthetic iron (III) oxide and raised the concentration in zero phosphate medium to 118  $\mu\text{M}$ . Therefore, the loss of viability of 36 in low phosphate concentration could have been due to an artifact of  $\text{Mn}^{2+}$  toxicity. This is unlikely since both P-starved 6 and 36 were found to tolerate  $\text{Mn}^{2+}$  concentrations up to 1 mM even under 1.5  $\mu\text{M}$  P concentrations.

The physiological reasons for the different tolerances and P sequestering abilities of R. trifolii strains 6 and 36 under low P conditions are unknown at this time. Recently, Poole and Hancock (1986) have shown that different kinds of channel forming proteins P are induced in the membrane of different types of bacteria for the purpose of P uptake in P-deficient environments. Furthermore, evidence has been obtained for leakage of inorganic phosphate and phosphorus-containing metabolites from a marine strain of Rhodotorula rubra under P-limited conditions (Robertson and Button, 1979). Such a phenomenon might also be an explanation for the loss of viability of 36 under low P conditions. Further studies are required to elucidate

the physiological reasons for the differences in behavior of 6 and 36 under phosphate limited conditions.

## CHAPTER III

Nodulation characteristics of two indigenous  
strains of Rhizobium trifolii under low  
phosphate conditions

## ABSTRACT

R. trifolii isolates 6 and 36 from Abiqua soil were shown to respond differently to low phosphate conditions in growth medium. In this study, the nodulation characteristics and rhizoplane dynamics of strains 6 and 36 on subterranean clover cv. 'Mt. Barker' were determined under low phosphate conditions at pH 5.5 and 6.5. A factorial experiment consisting of strains of bacteria [6 and 36] x phosphate status of the bacteria [phosphate-starved ( $B_0$ ) and luxury phosphate-grown ( $B_1$ )] x phosphate status of the plant growth medium (PGM) [zero phosphate ( $P_0$ ) and luxury phosphate ( $P_1$ )] x pH [5.5 and 6.5] was carried out. Nodules were enumerated on alternate days for 20 days, and rhizoplane populations were measured by immunofluorescence at 7 and 21 days post inoculation. Both 6 and 36 were able to nodulate subterranean clover cv. 'Mt. Barker' regardless of their own phosphate status or that of the PGM at both pH values. At pH 5.5,  $B_0$ -36 nodulated later (2 days) and produced fewer nodules than  $B_0$ -6 in  $P_0$ -PGM;  $B_1$ -36 performed no differently than  $B_1$ -6 under the same conditions. Nodulation by  $B_0$ -36 was improved (and not significantly different from  $B_0$ -6) in  $P_1$ -PGM at pH 5.5 or by increasing the pH of  $P_0$ -PGM to pH 6.5. Strain 6 did not differ in nodulation characteristics regardless of phosphate or pH. Both 6 and 36 had proliferated

substantially on the rhizoplane during the 7 day post inoculation period regardless of the phosphate or pH treatments. The number of generations achieved by  $B_0$ -36 was significantly less than  $B_1$ -36 regardless of the external phosphate status of PGM at the 7 day enumeration. The proliferation of  $B_0$ -36 on the rhizoplane was not influenced by pH. At the end of 21 days, all treatments had achieved similar number of generations and rhizoplane densities. Differences in nodulating abilities were not reflected in the rates of rhizoplane proliferation.

## INTRODUCTION

Production of legumes has often been attempted in soils suboptimum in their phosphate fertility status. Even though the effects of phosphate on legumes and their symbiotic effectiveness have long been studied, results obtained are equivocal. Gates (1974) and Gates and Wilson (1974) found that with the application of phosphate, Stylosanthes humilis showed substantial improvement in both the numbers of nodules and effectiveness of fixing nitrogen. Gates (1974) also showed that phosphate had a beneficial effect on the initiation of nodules. Increase in nodule numbers and mass on Trifolium alexandrium (berseem clover) as a result of phosphate addition was also observed by Kaushik and Singh (1969). On the other hand, both Trifolium subterraneum L. (subterranean clover) and Trifolium repens L. (white clover) showed no improvement in nodulation upon the addition of phosphate (Hallsworth et al., 1964). For Glycine max (soybean), nodulation responded differently to phosphate depending on the inoculum strain of Bradyrhizobium japonicum. For example, De Mooy and Pesek (1966) found that the number of nodules formed by soybean with the indigenous rhizobia in an Iowa soil increased with phosphate amendment but no such interaction was observed by Perkins (1924) in a Virginia soil presumably containing

different strains of B. japonicum. Recently, Singleton et al. (1985) found that soybeans inoculated with three of five B. japonicum strains responded to phosphate in terms of nodulation and effectiveness of nitrogen fixation.

Low phosphate conditions often accompany acid soils. Data from the studies of Thornton and Davey (1983) and Keyser and Munns (1979) showed that some strains of Rhizobium could tolerate low phosphate conditions better at high pH. Since very few reports have been published on this subject, the interaction of phosphate and acidity on growth and nodulation by Rhizobium is still unclear.

Previously, Dughri and Bottomley (1983) and Almendras and Bottomley (1985) have shown that indigenous strains of R. trifolii nodulate differently at different pH and phosphate levels respectively, yet, no definitive explanation was given for the observations. In the previous chapter, two indigenous strains of R. trifolii, 6 and 36, were shown to have different critical solution phosphate concentrations for growth and different responses to, and recovery from phosphate starvation. In this chapter, the objectives were to: (i) ascertain the nodulation characteristics of 6 and 36 on subterranean clover cv. 'Mt. Barker'; and (ii) determine the growth of the bacteria on the rhizoplane in response to phosphate and pH.

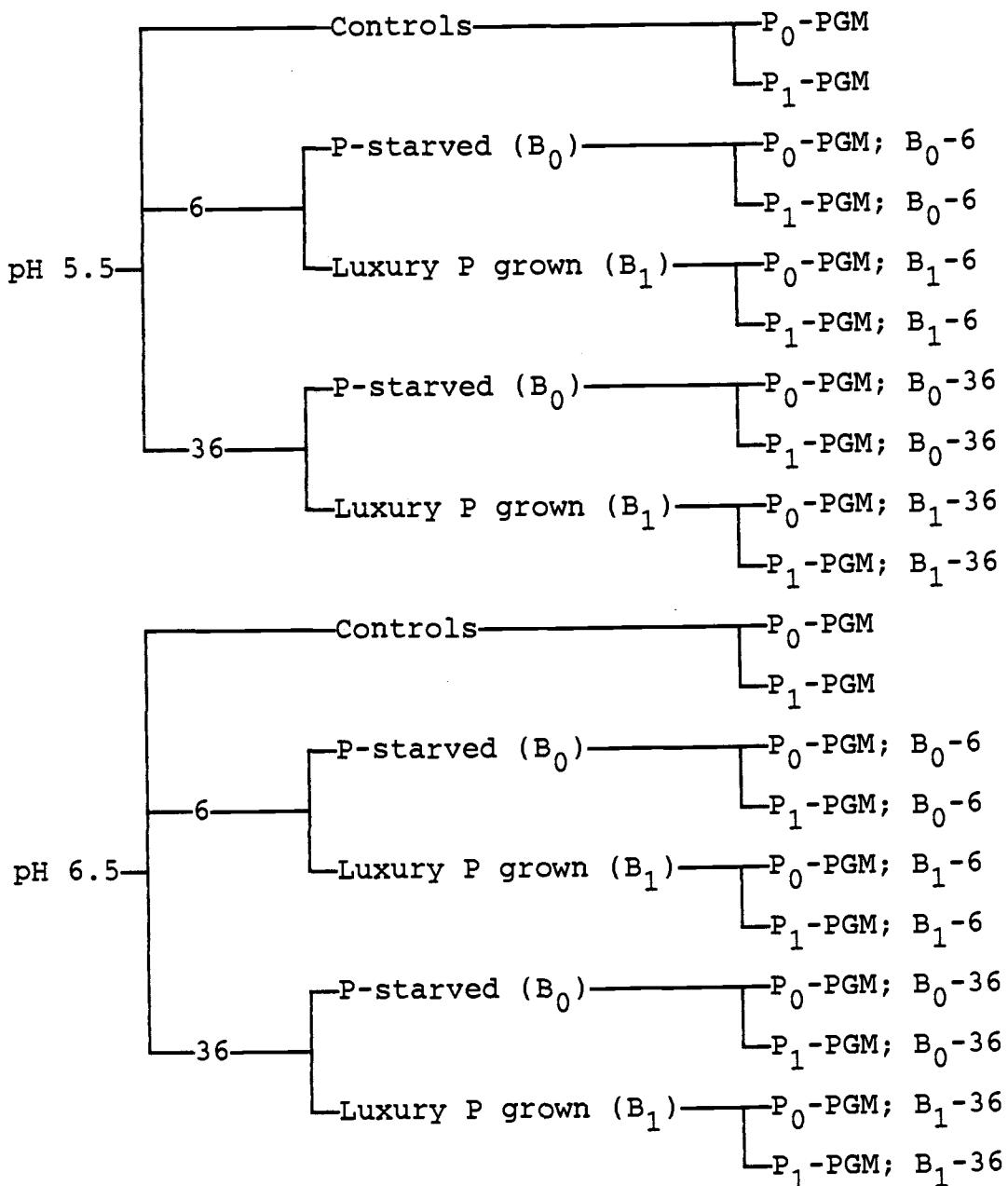
## MATERIALS AND METHODS

### Assessment and resolution of phosphate contamination.

Plant growth medium (PGM) used in this study was one-half strength of the mineral solution described elsewhere (Dughri and Bottomley, 1983). The pH of the solution was buffered with 10 mM MES at either 5.5 or 6.5.  $K_2SO_4$  (2.5 mM) was added to substitute for the potassium requirement of PGM without P addition (-PGM). Duplicate samples of (i) -PGM; (ii) -PGM exposed to a paper seedling support; (iii) -PGM exposed to iron (III) oxide powder (1:30 w/v), thereafter, abbreviated to,  $P_0$ -PGM; and (iv) -PGM exposed to both paper seedling support and oxide were analyzed for phosphate. They were steamed for 2h on two consecutive days. The samples were cooled and inorganic P contents were determined by the molybdenum blue method described in the previous chapter. Whatman No. 1 chromatographic paper and the original paper seedling support of the growth pouch were also analyzed for P contamination. The samples were digested in acidified ammonium persulphate solution and inorganic phosphate was determined by a standard addition method to overcome the interference of the colored background.

Evaluation of nodulating ability of strains 6 and 36 under different internal and external phosphate conditions. A factorial experiment involving treatments

of strains [6 or 36], P status of the bacteria [P-starved ( $B_0$ ) or grown in +PGG ( $B_1$ )], P status of PGM [ $P_0$ -PGM or  $P_1$ -PGM (PGM with 2.85 mM phosphate)] and pH [5.5 or 6.5] was carried out as described in the following flow chart.



(i) Inoculant preparation. The protocol for starving bacteria for phosphate was as described in the previous chapter. The  $B_0$ -bacteria were utilized upon reaching stationary phase. Cultures were harvested by centrifugation at 25,000 g for 15 min, cells were then washed twice with distilled water and diluted to approximately  $10^5$  cells  $\text{ml}^{-1}$  with distilled water.  $B_1$  bacteria were grown in +PGG at pH 5.5 or 6.5 two days prior to inoculation, and were washed and diluted in the same manner as the  $B_0$  cultures described above.

(ii) Preparation of seedlings and inoculation. The paper seedling supports of the commercially available growth pouches (Northrup King, St. Paul, MN) were substituted with replicas constructed from Whatman No. 1 chromatography paper (VWR). Seven ml of  $P_0$ -PGM or  $P_1$ -PGM (at pH 5.5 or 6.5 depending on treatment) were pipetted into each pouch. The pouches were steam-sterilized for 2h on two consecutive days prior to use. Surface-sterilized seeds of subterranean clover cv. 'Mt. Barker' (Vincent, 1970) were germinated as described elsewhere (Brockwell, 1980). Vigorous and uniform seedlings were chosen and three were transplanted aseptically to each of the growth pouches. Each seedling was inoculated with 10  $\mu\text{l}$  of the appropriate bacterial culture to achieve an inoculant size of about 1,000 cells per plant. Fifteen pouches were prepared for each treatment and were divided into 3

replicates of five pouches. Replicates were placed in a temperature controlled growth chamber described elsewhere (Demezas and Bottomley, 1986b). The root temperature of the plants was precisely controlled at 25°C by suspending the pouches in water baths and the shoot temperature was maintained between 23 and 28°C throughout the experiment. The numbers of nodules were counted at two day intervals up to 20d after inoculation. Sterile distilled water was added in quantities just sufficient to keep the Whatman paper saturated.

Growth of 6 and 36 on rhizoplane. The experiment was set up as in the nodulation experiment with the following variations. Four subterranean clover seedlings were grown in each pouch and the inoculant size was about  $10^4$  cells per plant. Three replicates were used for every treatment and samples for rhizoplane counts were taken 7 and 21d after inoculation.

Determination of rhizoplane populations. Only vigorous root systems were chosen for rhizoplane extraction. Nodules were removed from the roots to prevent contamination. Roots were shaken in 50 ml of 0.15 M phosphate buffer (pH 7.2) with 0.5% (v/v) phenol and 5g glass beads (3 mm diameter) for 15 min on a wrist action shaker. After extraction, roots were recovered from the extracts and their dry weights were determined.

Portions (5 or 10 ml) of extracts were filtered and the

populations of 6 and 36 were determined by immunofluorescence as described elsewhere (Demezas and Bottomley, 1986b).

Determination of phosphatase activities. Bacterial cultures of 6 and 36 were prepared in P-supplemented (2.85 mM P) defined growth medium at pH 5.5, and samples of the 1st P-runout cycle were obtained by transferring 0.1 ml of the luxury P-grown bacterial culture to P-depleted medium (pH 5.5) and grown until constant cell density was reached. One-tenth of a ml of the first P-runout bacterial culture was backtransferred to P-depleted medium again and grown to constant turbidity to complete the 2nd P-runout cycle. One-half ml of samples were added to dicyclohexylammonium p-nitrophenyl phosphate ( $8 \text{ mg ml}^{-1}$ ) in 5.5 ml modified universal buffer (Tabatabai, 1982) at pH 5, 5.5, 6.5, 9, and 11. Four ml 0.5 M NaOH solution were used to stop the phosphatase activity after incubating at  $30^\circ\text{C}$  for 20 to 60 min and absorbance of p-nitrophenol measured at 400 nm. Phosphatase activity was expressed as nmoles p-nitrophenol produced ( $\text{mg dry wt}$ ) $^{-1} \text{ min}^{-1}$ .

Symbiotic competence of 6 and 36 after exposing to P stress.  $B_0$ -6 and  $B_0$ -36 were prepared as described previously. Portions of the cultures were plated onto YMA and incubated at  $27^\circ\text{C}$ . Twenty five colonies of each strain were harvested and inoculated onto subterranean clover seedlings growing on mineral nutrient agar slants

(Dughri and Bottomley, 1983). At the same time, cells of 6 and 36 grown in normal P-containing medium were also used to inoculate subterranean clover seedlings in replicates of 5 to serve as controls. Plants were grown under greenhouse conditions as described elsewhere (Dughri and Bottomley, 1983) and nodulation was examined two weeks after inoculation. Shoot dry weights were determined on plants harvested 6 weeks after inoculation.

## RESULTS

Both the plant growth medium and the paper supports within the pouches contained measurable levels of phosphate (Table 5). Whatman No. 1 chromatographic paper was virtually free of P contamination and in addition contained only 1.5 umoles N per growth pouch support. Seedling supports were constructed from this material, and contaminating phosphate in the plant growth medium was removed effectively by pretreating with iron (III) oxide powder.

No differences in symbiotic competency were observed between inocula originating from strains (6 or 36) grown under P-starved ( $B_0$ ) or luxury-P ( $B_1$ ) conditions. All of the 25 isolates of both  $B_0$ -6 and  $B_0$ -36 inoculated onto subterranean clover seedlings were capable of nodulation. Mean shoot dry weights of the plants inoculated with  $B_0$ -6 and  $B_0$ -36 isolates were not significantly different from those infected with  $B_1$ -6 and  $B_1$ -36 (Table 6).

Both 6 and 36 were able to nodulate subterranean clover cv. 'Mt. Barker' regardless of their own phosphate status or that of the plant growth environment at pH 5.5 (Fig. 6) and 6.5 (Fig. 7). Although the numbers of nodules formed by 20 day post inoculation were similar across all treatments, significant differences were observed (i) between different

treatments of the same strain, and (ii) between strains exposed to the same treatment. For example, at pH 5.5, appearance of nodules by  $B_0$ -36 in  $P_0$ -PGM was delayed by 2 to 4 days when compared with other treatments which took 6 to 8 days for nodulation (Fig. 6a-d). However, when cells of  $B_0$ -36 were exposed to seedlings developing in  $P_1$ -PGM at pH 5.5, the time to the appearance of nodules was shorter and the number of nodules formed by day 20 was significantly greater ( $P=0.05$ ) than in  $P_0$ -PGM (Fig. 6b). Treatment differences were not observed with  $B_1$ -36 which nodulated at the same time and resulted in the same number of nodules regardless of the P status of the environment (Fig. 6c-d).

Strain differences within the same treatment were observed between  $B_0$ -6 and  $B_0$ -36 in  $P_0$ -PGM at pH 5.5.  $B_0$ -6 showed superior nodulating ability than  $B_0$ -36 in both the time to nodule appearance and the number of nodules which had formed on 5 of the 7 sampling dates ( $P=0.05$ ) (Fig. 6a). In  $P_1$ -PGM,  $B_0$ -6 produced significantly ( $P=0.05$ ) fewer nodules than  $B_0$ -36 only at day 20 although the performance of  $B_0$ -6 per se was not different than in  $P_0$ -PGM. Generally, 6 formed nodules on the subterranean clover roots at day 8 regardless of the P regime or pH.

At pH 6.5, nodule appearance and rate of nodule formation by both organisms were similar (Fig. 7). An influence of pH was apparent with regard to 36. P-

deficient cells of the latter ( $B_0$ -36) nodulated earlier (8d) (Fig. 7a) than those at pH 5.5 (10d) (Fig. 6a) in  $P_0$ -PGM. However, no significant differences between accumulation of nodules by either strain were observed in any pH 6.5 treatment in contrast to pH 5.5.

The magnitudes of rhizoplane populations indicated that both 6 and 36 had proliferated substantially during the 7 day post inoculation period (5.4 to 9.2 generations) regardless of P or pH treatments (Table 7). At pH 5.5,  $B_1$ -36 proliferated significantly ( $P=0.05$ ) more (8.1 to 8.5 generations) than  $B_0$ -36 (5.7 to 6.4 generations) regardless of the P status of the nutrient solution. In the case of 6, no differences were apparent at the same pH. The number of generations ranged from 7.1 to 8.1 for  $B_0$ -6 and  $B_1$ -6 respectively.

At pH 6.5, in contrast to pH 5.5, no significant differences were observed between the numbers of generations completed by  $B_0$ -36 and  $B_1$ -36. The average generations established during the 7 day post inoculation period by 6 and 36 were 8.5 and 6.0 respectively and in general, no differences were found between treatments of the same strain. Furthermore, the effect of pH on the two organisms was not significant with respect to the number of generations.

At 21 days, the average number of generations of all the treatments was 8.7 and significant treatment effects were not apparent. With few exceptions, the

increases in the number of generations between 7 and 21 days were not significant. One of the exceptional cases was  $B_0$ -36 at pH 5.5 where significant proliferation had occurred during this period regardless of the external P concentration (Table 8).

From the 7 day rhizoplane count, there was a general trend for lower cell densities to be observed for P-starved bacteria of either 6 or 36 in comparison to luxury P-grown cells (even though significant differences were only found on 36 grown at pH 5.5) (Fig. 8). No significant effects were observed on the bacterial densities with different P status in the nutrient solution. With the exception of the superior growth of the  $B_1$ -6 in  $P_1$ -PGM at pH 6.5, the increase of pH from 5.5 to 6.5 did not significantly increase the cell densities of the bacteria. At 21 days, the density differences between  $B_0$ - and  $B_1$ -36 at pH 5.5 had disappeared. With few exceptions, rhizoplane densities of 6 and 36 had dropped at day 21 (Fig. 8). In general, rhizoplane enumeration at 21 days revealed there had been a slow increase in population of bacteria on the rhizoplane between the 7 and 21d rhizoplane count. However, no increase in the number of  $B_1$ -6 was found on the rhizoplane of the subterranean clover roots grown in  $P_1$ -PGM at pH 6.5. This caused a few fold decline in rhizoplane density in this treatment.

Phosphatase activity was found to be extremely

high in both 6 and 36 under P-deficient conditions (Table 9), and was about two hundred fold higher in magnitude after two cycles of P-depleted growth than in P-sufficient medium. pH also influenced the activity of the phosphatase which was significantly greater ( $P=0.05$ ) at pH 6.5 than at pH 5.5 after one cycle of P-depleted growth and significantly greater ( $P=0.05$ ) at pH 5.5 than pH 5.0 after two cycles of depletion.

Figure 6. Nodulation characteristics of R. trifolii strains 6 and 36 at pH 5.5. Symbols ● and ○ represent strains 6 and 36 respectively. a. nodulation of  $B_0$  bacteria in  $P_0$ -PGM; b.  $B_0$  bacteria in  $P_1$ -PGM; c.  $B_1$  bacteria in  $P_0$ -PGM; and d.  $B_1$  bacteria in  $P_1$ -PGM. Vertical bars represent standard deviations (SD) at the 95% confidence level. No vertical bar indicates SD is smaller than the symbol.

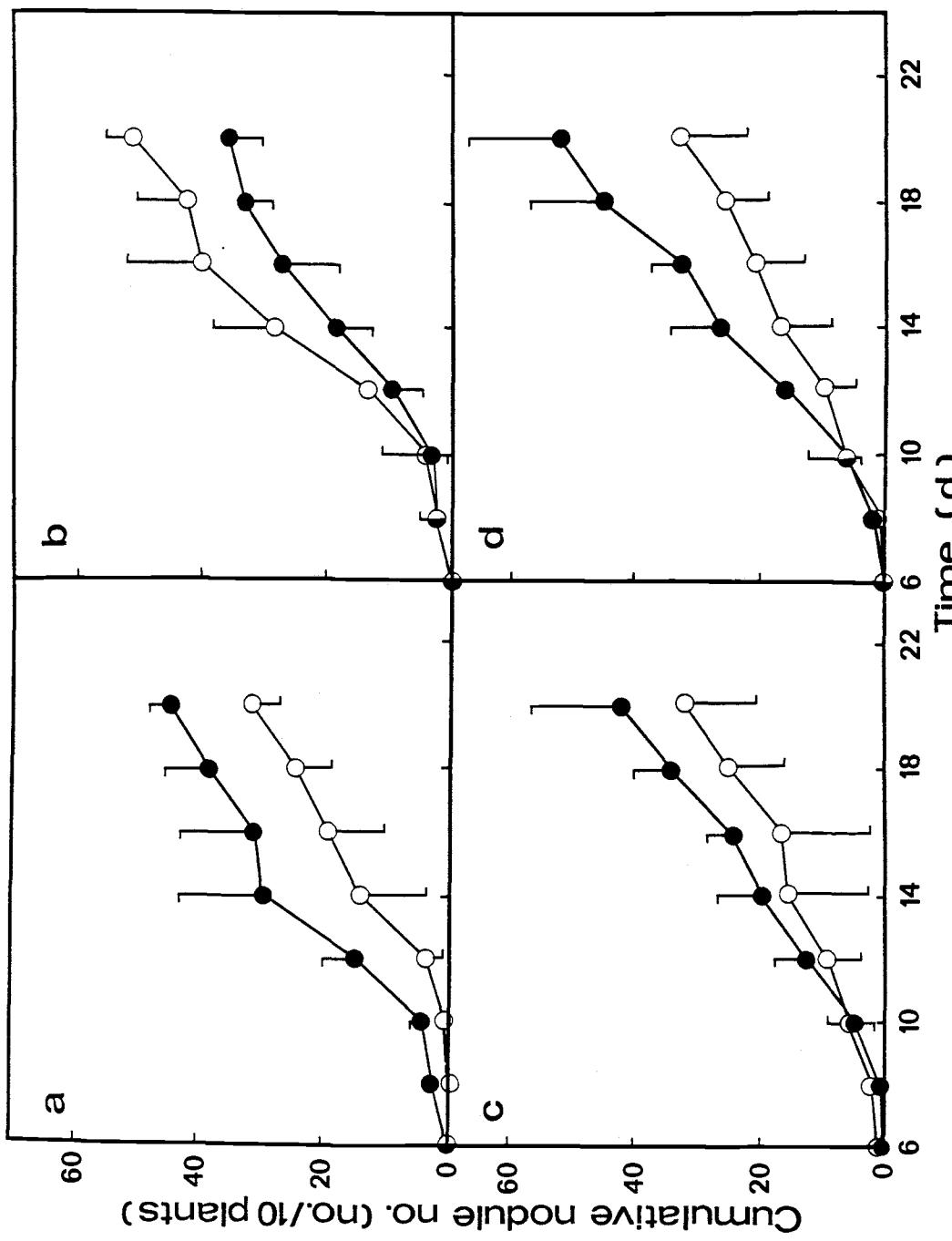


Figure 6

Figure 7. Nodulation characteristics of R. trifolii strains 6 and 36 at pH 6.5. Symbols ● and ○ represent strains 6 and 36 respectively. a. nodulation of  $B_0$  bacteria in  $P_0$ -PGM; b.  $B_0$  bacteria in  $P_1$ -PGM; c.  $B_1$  bacteria in  $P_0$ -PGM; and d.  $B_1$  bacteria in  $P_1$ -PGM. Vertical bars represent standard deviations (SD) at the 95% confidence level. No vertical bar indicates SD is smaller than the symbol.

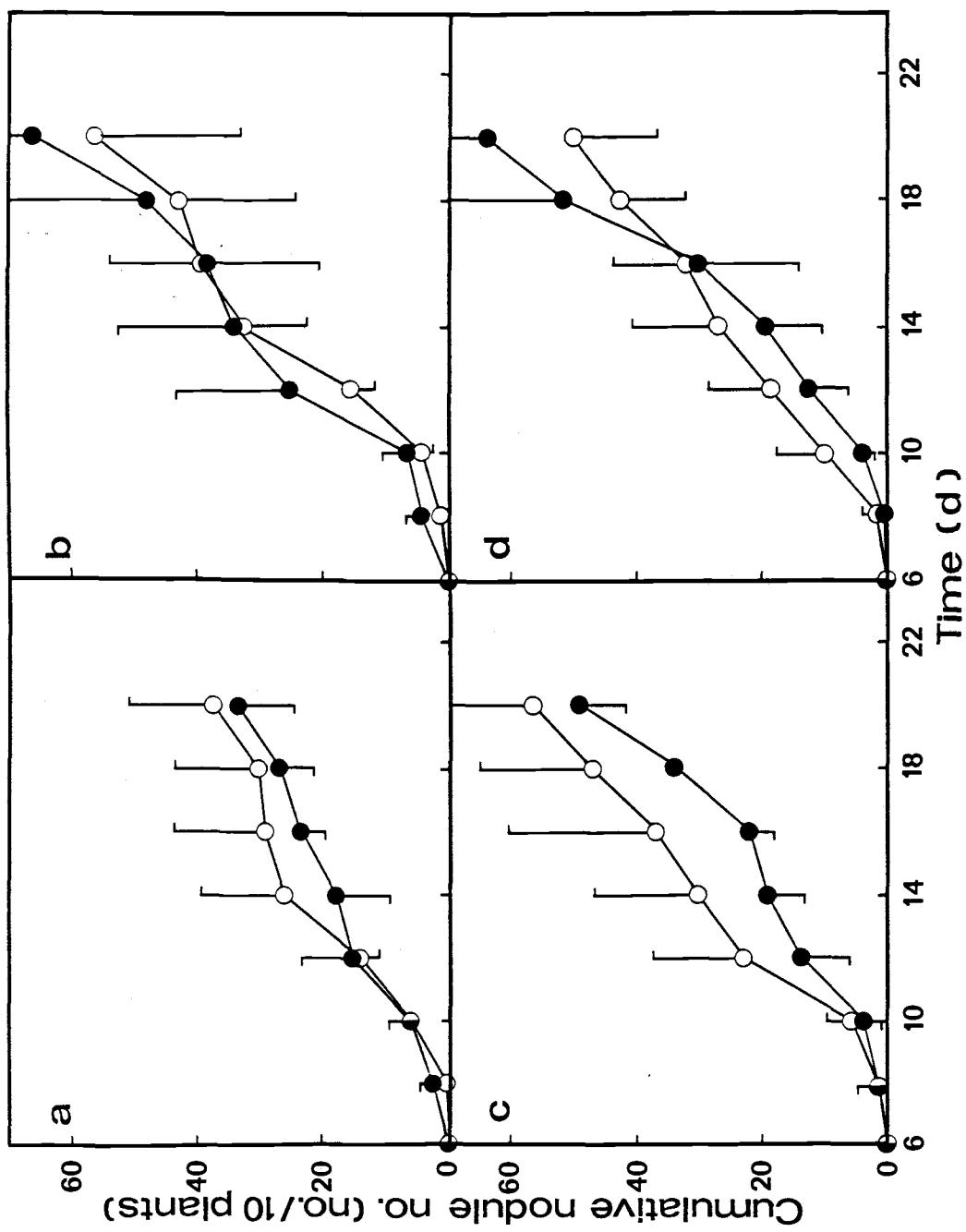


Figure 7

Figure 8. Rhizoplane density dynamics of R. trifolii strains 6 and 36 at (A) pH 5.5 and (B) pH 6.5.  $B_0$  and  $B_1$  represent phosphate-starved and luxury phosphate grown bacteria respectively.  $P_0$  and  $P_1$  represent zero and 2.85 mM phosphate containing plant growth medium. Control treatments were not inoculated. Vertical bars represent standard deviations at the 95% confidence level.

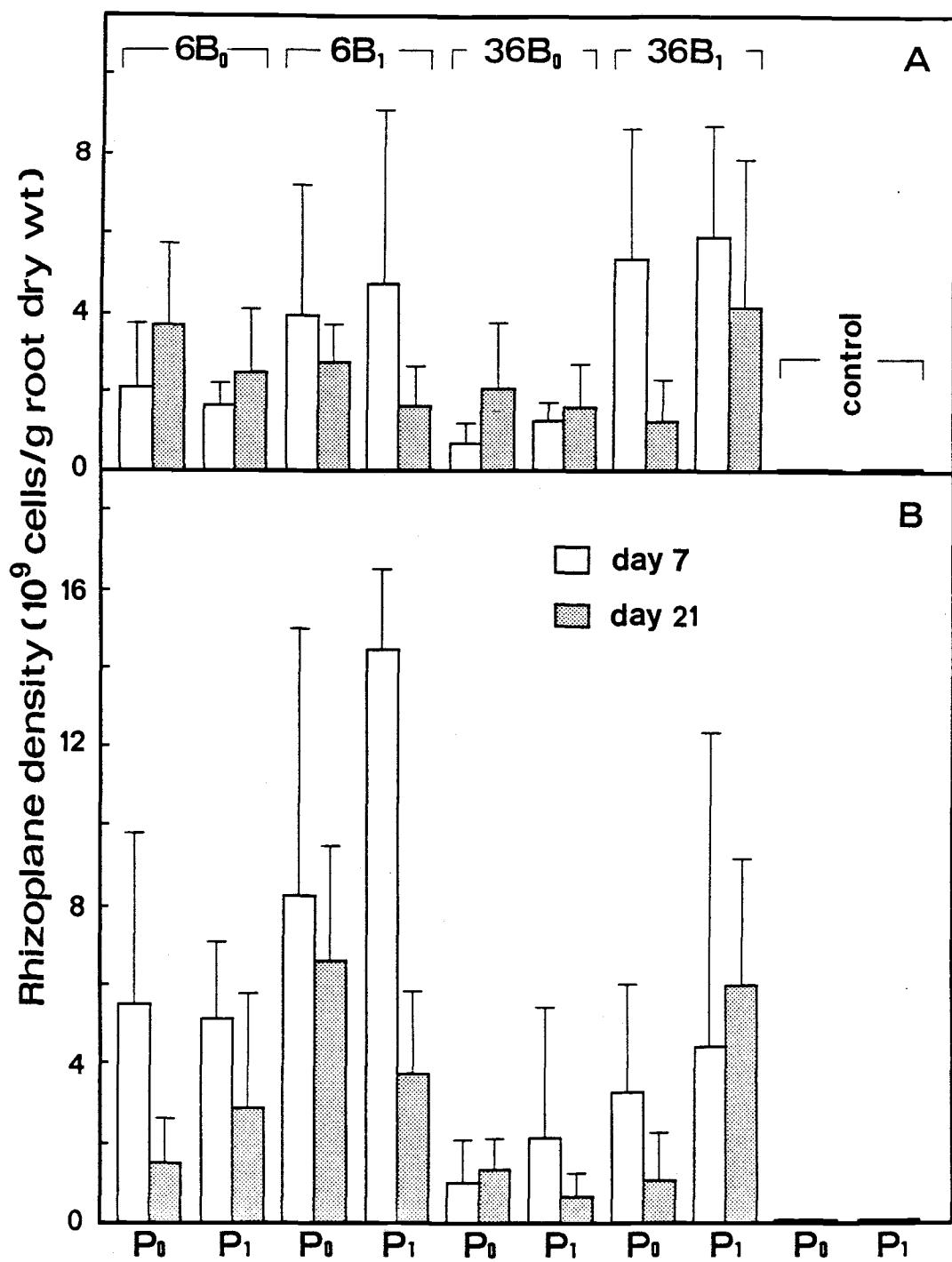


Figure 8

Table 5. Phosphate contamination of both plant growth medium (PGM) and the growth pouch support (GPS).

Material analyzed	Phosphate contamination	
	PGM — uM —	GPS — umol GPS <sup>-1</sup> —
PGM alone	0.2 ± 0.0 <sup>†</sup>	-
PGM + GPS	31.9 ± 7.1 <sup>‡</sup>	-
PGM + Fe <sub>2</sub> O <sub>3</sub> powder	0.0 ± 0.0	-
PGM + GPS + Fe <sub>2</sub> O <sub>3</sub> powder	0.0 ± 0.0	-
GPS original <sup>§</sup>	-	0.38 ± 0.21
GPS constructed of Whatman No. 1 chromatographic paper <sup>¶</sup>	-	0.00 ± 0.08

<sup>†</sup>Standard deviation at the 95% confidence level.

<sup>‡</sup>Calculated by relating the absolute quantity of phosphate contamination of the GPS to the volume (7 ml) of PGM typically required to saturate the GPS for plant growth.

<sup>§</sup>3.2 g per sheet.

<sup>¶</sup>2.3 g per sheet.

Table 6. Symbiotic competency of strains 6 and 36 on subterranean clover cv. 'Mt. Barker' after exposure to P-starvation conditions.

Symbiotic parameter	Strains			
	P status of the bacteria <sup>†</sup>			
	6	36	P-sufficient	P-starved
Proportion of isolates nodulating (%)	100	100	100	100
Dry shoot yield (mg plant <sup>-1</sup> )	37.5 ± 8.8 <sup>‡</sup>	32.9 ± 1.8	27.1 ± 3.3	31.5 ± 2.4

<sup>†</sup>See materials and methods for bacterial culture conditions. Twenty five and five isolated colonies were evaluated to represent the P-starved and P-sufficient cultures of each strain respectively.

<sup>‡</sup>Standard deviation at the 95% confidence level.

Table 7. Number of generations completed by *R. trifolii* strains 6 and 36 on subterranean clover rhizoplanes in 7 days.<sup>†</sup>

Bacterial strains	pH							
	5.5				6.5			
	P status of inoculant bacteria							
	B <sub>0</sub>	B <sub>1</sub>	B <sub>0</sub>	B <sub>1</sub>	B <sub>0</sub>	B <sub>1</sub>	B <sub>0</sub>	B <sub>1</sub>
	P <sub>0</sub>	P <sub>1</sub>	P <sub>0</sub>	P <sub>1</sub>	P <sub>0</sub>	P <sub>1</sub>	P <sub>0</sub>	P <sub>1</sub>
	No. of generations <sup>‡</sup>							
6	7.5+1.7 <sup>§</sup>	7.1+0.5	7.6+1.8	8.1+1.6	8.4+1.0	8.1+0.5	8.1+0.7	9.2+0.1
36	5.7+0.9 <sup>a¶</sup>	6.4+0.3 <sup>b</sup>	8.1+0.8 <sup>a</sup>	8.5+0.6 <sup>b</sup>	5.4+2.2	5.7+2.3	6.7+1.4	6.2+2.8

<sup>†</sup>See materials and methods for details of experimental design, procedures, and definitions of symbols.

<sup>‡</sup>Numbers of generations calculated from differences between the initial inoculant sizes and the populations determined on the rhizoplanes at day 7.

<sup>§</sup>Standard deviation at the 95% confidence level.

<sup>¶</sup>Pairs of values for the same strain followed by the same letter are significantly different ( $P=0.05$ ) as determined by t-test comparisons.

Table 8. Number of generations completed by *R. trifolii* strains 6 and 36 on subterranean clover rhizoplanes at pH 5.5.

Strains									
	6				36				
	P status of inoculant bacteria				P status of PGM				
	B <sub>0</sub>	B <sub>1</sub>	B <sub>0</sub>	B <sub>1</sub>	P <sub>0</sub>	P <sub>1</sub>	P <sub>0</sub>	P <sub>1</sub>	
Time (d) (post inoculation)									
	P <sub>0</sub>	P <sub>1</sub>	P <sub>0</sub>	P <sub>1</sub>	P <sub>0</sub>	P <sub>1</sub>	P <sub>0</sub>	P <sub>1</sub>	
No. of generations <sup>‡</sup>									
7	7.5 <sub>±</sub> 1.7 <sup>§</sup>	7.1 <sub>±</sub> 0.5	7.6 <sub>±</sub> 1.8	8.1 <sub>±</sub> 1.6	5.7 <sub>±</sub> 0.9 <sup>a¶</sup>	6.4 <sub>±</sub> 0.3 <sup>b</sup>	8.1 <sub>±</sub> 0.8 <sup>a</sup>	8.5 <sub>±</sub> 0.6 <sup>b</sup>	
21	10.1 <sub>±</sub> 1.4	9.5 <sub>±</sub> 0.6	9.2 <sub>±</sub> 0.3	8.1 <sub>±</sub> 0.7	9.0 <sub>±</sub> 1.3	8.6 <sub>±</sub> 1.0	7.7 <sub>±</sub> 1.7	9.1 <sub>±</sub> 1.3	
Significance <sup>††</sup>	NS	*	NS	NS	*	*	NS	NS	

<sup>†</sup>See materials and methods for details of experimental design, procedures, and definitions of symbols.

<sup>‡</sup>Number of generations calculated from the differences between the initial inoculant sizes and the populations determined on rhizoplanes at specific time.

<sup>§</sup>Standard deviation at the 95% confidence level.

<sup>¶</sup>Pairs followed by the same letter at the 7d sampling date are significantly different ( $P=0.05$ ) as determined by t-test comparisons.

<sup>††</sup>Designates whether the number of generations completed by 21d is significantly greater ( $P=0.05$ ) than at 7d for a specific treatment.

Table 9. Phosphatase activities of 6 and 36 under different phosphate conditions and assayed at different pH values.

pH	Strains					
	6		P status of the bacterial growth medium		36	
	P-sufficient <sup>†</sup>	1st P-runout <sup>§</sup>	2nd P-runout <sup>¶</sup>	P-sufficient	1st P-runout	2nd P-runout
nmol p-nitrophenol (mg dry wt) <sup>-1</sup> min <sup>-1</sup>						
5.0	1	42	54	1	30	30
5.5	1	54	180	1	38	80
6.5	1	235	230	1	190	260
9.0	0	22	57	0	44	58
11.0	0	16	15	0	26	33
LSD <sub>(0.05)</sub>	0.1	53.4	51.6	0.4	42.3	15.1

<sup>†</sup>See materials and methods for details of experimental procedures.

<sup>‡</sup>Glucose-glutamate defined medium with 2.85 mM phosphate.

<sup>§</sup>Cell transferred from P-sufficient to P-depleted growth medium (contained 0.58 uM contaminated phosphate) and grown until constant cell density was reached.

<sup>¶</sup>Cells from 1st P-runout cycle were transferred to another bath of P-depleted medium and grown until constant cell density was reached.

## DISCUSSION

Very little work has been done on the nodulation of roots by severely P-starved rhizobia under conditions of P-depleted plant nutrient solution. Our data suggest that neither the status of internal P nor external inorganic P are critical for nodulation to occur. At pH 5.5, nodulation by  $B_0$ -36 was improved significantly by the presence of phosphate in the PGM (Fig. 6a, b). Previously,  $B_0$ -36 was found to grow poorly under P-limited conditions (see chapter 2). Therefore, it is possible that  $B_0$ -36 requires a longer time to adapt to the plant root as a limited source of either organic or inorganic P. With the presence of inorganic phosphate in the PGM,  $B_0$ -36 can recover more quickly and the delay of nodulation disappears. In the case of 6, effective nodulation occurred regardless of the P status of the bacteria. This is consistent with those data reported in chapter 2 where 6 could tolerate P stress and commence proliferation at much lower solution phosphate concentrations than 36.

In  $P_0$ -PGM, nodulation of  $B_0$ -36 was also improved at pH 6.5 and it was no longer significantly inferior to 6. pH effects on the nodule occupancy by indigenous serogroups 6 and 36 have been studies by Dughri and Bottomley (1983) and Almendras and Bottomley (1985). Both studies showed that increasing soil pH without any

addition of phosphate increased the ability of 36 to nodulate. It is possible to invoke a role of the phosphatase activity of  $B_0$ -36 which was three to nine times higher at pH 6.5 than at pH 5.0. Perhaps improvement in phosphatase activity at higher pH facilitated the ability of  $B_0$ -36 to hydrolyse organic phosphate released from the roots into the rhizosphere.

It is also of interest to speculate whether the 2 to 4d delay in nodulation by  $B_0$ -36 at pH 5.5 may be relevant to the outcome of competitive nodulation in soil. According to Kosslak and Bohlool (1985), preexposure of soybean to a B. japonicum strain for only 24h prior to a second strain or soil rhizobia greatly enhanced the nodulating ability of the primary inoculant strain. If representatives of serogroup 36 are indeed P-stressed in unamended Abiqua soil, a delay in nodulation relative to other indigenous Rhizobium, such as serogroup 6, may be sufficient to exclude 36 from the majority of nodules.

Rhizoplane enumeration showed that both 6 and 36 could proliferate very well under stringently low inorganic phosphate conditions on the rhizoplane (Drew and Nye, 1970; Bhat and Nye, 1974a, b). It was surprising since data presented in chapter 2 showed that both the organisms were not able to proliferate in nutrient medium containing  $< 0.28 \mu\text{M}$  phosphate. By inference, perhaps the bacteria were obtaining organic

phosphate from the roots of the subterranean clover through utilizing their acid phosphatases. The observations of Barber and Loughman (1967) should not be overlooked, however, with the possibility that 6 and 36 might also obtain inorganic P leaking from the plant roots under extremely low solution phosphate conditions.

Both 6 and 36 achieved most of their growth in the first week and slowed down thereafter resulting in a decline in bacterial density on the rhizoplane of most treatments by the third week. Similar findings were made by Reyes and Schmidt (1981) with slow growing B. japonicum. It seems that the proliferation of rhizobia on the rhizoplane of a healthy growing plant cannot always keep up with the expansion of the host root. In some cases, densities of bacteria could be maintained beyond the first week and they were often the  $B_0$  bacteria (6 or 36) at pH 5.5. Either, external P remained unused as a result of the slow growth during the first week, or the plants continue to excrete P between 7 and 21d only sufficient to allow proliferation of cells until a maximum population is reached.

In our studies, the rhizoplane densities of the bacteria, measured at 7d, did not correlate with their nodulating characteristics. For example, the superior nodulating ability of  $B_0$ -6 over  $B_0$ -36 in  $P_0$ -PGM at pH 5.5 was not reflected in their relative rhizoplane densities at 7d. Furthermore, even with a significantly

higher rhizoplane density,  $B_1$ -6 did not nodulate better than  $B_0$ -36 at pH 6.5. Therefore, the number and the proliferation of bacteria on the rhizoplane can not be a true indicator for the successfulness of infection. Studies of Moawad et al. (1984) and Moawad and Bohlool (1984) also showed that colonization of the host rhizosphere by specific strains of Rhizobium was no guarantee for domination of nodulation.

The possibility that some of the cells of 36 might have lost symbiotic competence during P starvation was not found since all isolates of  $B_0$ -6 and  $B_0$ -36 showed no problem in nodulation (Table 6). Presumably the sym plasmid and nodulation genes of the two strains are rather stable to extremely P-limited growth conditions.

Very few studies have been carried out under controlled environmental conditions in an attempt to repeat observations made under soil conditions. This study provides a link between differences in P sequestering ability and nodulation capabilities by members of indigenous serogroups 6 and 36, and which relates to findings actually made with soil grown legumes.

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