INFLUENCE OF WATER POTENTIAL ON THE ENUMERATION OF *BRADYRHIZOBIUM* SP (CICER) BY Tn5 MUTANTS (Km⁻) AND FLUORESCENT ANTIBODY[1]

MILTON A.T. VARGAS[2] and DAVID F. BEZDICEK[3]

ABSTRACT - Tn5 induced insertion mutants were generated in *Bradyrhizobium* sp (Cicer) by mating with *Escherichia coli* MV-12 carrying the suicide plasmid pGS9. One Tn5 mutant of strain A8 was selected, inoculated in nonsterile soil at three water potentials (-0.033, -0.3 and -3 MPa) and enumerated for survival by plate count using kanamycin (Km) amended medium and by the fluorescent antibody (FA). Survival, as determined by plate count and FA was optimum at -0.3 MPa where the strain remained viable at near the initial inoculum level for at least 55 days. At the other two water potentials, bradyrhizobial viable counts decreased sharply, particularly at -3 MPa. Initially, FA and plate counts were similar, but 10 days after inoculation, estimates by FA were two log units higher than by plate count for soils held at -3 MPa. The discrepancy between the two methods may be due to identification of fluorescent active dead cells or failure of alive, but stressed cells to grow on the artificial medium. Under minimum water stress (-0.033 and -0.3 MPa water potential), results with both methods were similar. These results show that Tn5 mutagenesis can be used to mark bacteria and to conduct effective ecological studies. Additionally, they indicate that FA enumeration tends to overestimate the bradyrhizobial population under some dry soil conditions that reduce the microbial activity in soil.

Index terms: *Bradyrhizobium*, Tn5, transposon, ecology.

EFEITO DO POTENTIAL DE ÁGUA NO SOLO NA POPULAÇÃO DE *BRADYRHIZOBIUM* sp (CICER) AVALIADA ATRAVÉS DE MUTANTES CONTENDO O TRANSPOSON Tn5 (Km⁻) E IMUNO-FLUORESCÊNCIA

RESUMO - Foram obtidos mutantes de *Bradyrhizobium* sp com o transposon Tn5 através de conjugação com a estirpe MV-12 de *Escherichia coli* contendo o plasmídio suicida pGS9, com estirpes de *Bradyrhizobium* de grão-de-bico (*Cicer arietinum* L.). Um dos mutantes Tn5 da estirpe A8 foi selecionado, inoculado em solo não estéril submetido a três tensões de água: -0.033, -0.3 e -3 MPa, e feito o acompanhamento de sua população através de contagem em placas e através de imunofluorescência (FA). O maior índice de sobrevivência da estirpe A8 foi observado em -0.3 MPa, mantendo-se a população ao nível inicial até pelo menos 55 dias. Nos dois outros níveis de tensões de água, a população de *Bradyrhizobium* sofreu um decréscimo acentuado com o tempo, particularmente no nível de -3 MPa. Inicialmente, os valores obtidos através da contagem em placas e FA foram similares, mas 10 dias após a inoculação, as contagens através do FA foram cerca de 100 vezes superiores aos da contagem em placas nas amostras mantidas a 3 MPa. A discrepância entre os dois métodos poderia ser atribuída à contagem de células mortas, mas ainda mantendo propriedades antigênicas intactas, ou incapacidade de células vivas mas estressadas, de crescerem no meio utilizado. Em solos com teores mais elevados de água (-0.033 e -0.3 MPa) os resultados obtidos pelos dois métodos foram similares. Os resultados indicam que mutações induzidas pelo transposon Tn5 podem ser utilizadas em estudos ecológicos. Também sugerem que o método de FA parece superestimar a população de *Bradyrhizobium* no solo sob condições de baixa umidade, que reduza a atividade microbiana no solo.

Termos para indexação: *Bradyrhizobium*, Tn5, transposon, ecologia.

INTRODUCTION

In the absence of its host legume, *Bradyrhizobium* sp is a common soil microorganism and as such it may be as affected by the soil environmental stress as are all soil organisms.

Research indicates that bradyrhizobial survival is affected by soil moisture levels (Pena-Cabriales & Alexander 1979, Osa-Afiana & Alexander 1982a, b; Hartel & Alexander 1985; Fuhrmann et al. 1986) that eventually can affect nodulation of the host legume in field soil (Marshall 1964, Chatel & Parker 1973, Herridge et al. 1987).

Ecological studies on the effect of moisture levels on bradyrhizobium survival has used spontaneous antibiotic resistant strains (Pena-Cabriales & Alexander 1979, Osa-Afiana & Alexander 1982a, b; Hartel & Alexander 1985) or experiments carried out under sterile conditions Rensburg & Strijdon 1980, Mary et al.. 1985, Fuhrmann et al. 1986). Either of these methods have serious limitations; results otained uner axenic conditions cannot be extrapolated to the field, and spontaneous mutants may differ phenotypically from the parent strain (Jones & Bromfield 1978, Turco et al. 1986). The selection methods used to obtain mutants with high levels of antibiotic resistance may allow undesirable mutations that affect certain functions of the cell (Novick 1980), and alter the overall organisms environmental competitiveness.

The fluorescent antibody (FA) technique has been widely used to study bradyrhizobial ecology under conditions in the soil presumed to be nonstressed, since Schmidt and co--workers (Schmidt et al. 1968, Bohlool & Schmidt 1970) demonstrated its efficacy for direct enumeration of *Bradyrhizobium* strains in the soil. However, under certain soil stress conditions such as dryness (Bezdicek & Donaldson 1981) or heat (Kennedy & Wollum, 1988) the FA technique tends to overestimate the bradyrhizobial population.

Transposon mutagenesis has been used extensively in genetic manipulation and characterization of *Bradyrhizobium* species (Noel et al. 1984, Vesper et al. 1987) but only recently has transposon-associated antibiotic resistance been used as as selective marker in bradyrhizobial ecology (Fredrickson et al. 1988). Tn5 mutagenesis in *Bradyrhizobium* species is based on the insertion of a

transposable element Tn5 through a suicide plasmid (Selvaraj & Iyer 1983), into the bradyrhizobial chromosome. The inserted Tn5 sequence in *Bradyrhizobium* species expresses resistance to some antibiotics such as kanamycin, neomycin and streptomycin (Selvaraj & Iyer 1983).

The objectives of this study were: (i) to evaluate the efficacy of the transposable element Tn5 as a kanamycin-resistant marker in bradyrhizobial ecological studies, and (ii) to determine the effect of soil moisture stress levels on survival of a Tn5 mutant of *Bradyrhizobium* sp (Cicer) using the FA and the kanamycin resistant marker techniques for bradyrhizobial enumeration.

MATERIALS AND METHODS

Bacterial strains

Bradyrhizobium sp (Cicer) strains 27a3, 27a8 and 27a16 (hereafter called A3, A8 and A16, respectively) were obtained from the culture collection of The Nitragin Co. (Milwaukee, WI). *Rhizobium leguminosarum* biovar *phaseoli* Vik1, was obtained from E.L. Schmidt (University of Minnesota) and *R. leguminosarum* biovar *phaseoli* Kim5 was from the laboratory of D.F. Bezdicek (Washington State University).

The transposon donor was *Escherichia coli* MV12 (leu⁻ thr⁻ thi⁻ trp⁻ thy⁻) obtained from M. Kahn (Washington State University) transformed with the plasmid vector pGS9 containing Tn5, from V. Iyer (Carlerton University).

Media and growth conditions

Rhizobium strains were maintained on yeast extract mannitol agar (YMA) slants and grown to late log phase at 30°C in nutrient broth of the same medium. *E. coli* was maintained on Luria-Bertani (LB) agar slants (Maniatis et al. 1982) and grown in LB broth with $100 \, \mu g \, mL^{-1}$ of chloramphenicol (Cm) at 37°C.

Bacterial matings

Log phase cultures of donor and recipient cells were pelleted by centrifugation (8,000 x g for 5 min. in a Sorval microfuge), washed twice in sterile filtered saline (0.85% NaCl) and resuspended to

about 10^9 cells mL^{-1}. Donor and recipient cells were mixed (0.1 mL each) and placed on sterile (45-mm diameter) nitrocellulose membrane filters on YMA and incubated for 16 h at 30°C. Selection of putative transconjugants was made on *Rhizobium* minimal salts (RMS) containing per L: 15 g agar; 10 g mannitol; 1.0 g K_2HPO_4; 1 g KH_2PO_4; 0.5 g NH_4Cl; 0.25 g Na_2SO_4; 0.25 g $MgCl_2$; 0.1 g $CaCl_2.2H_2O$; 0.01 g $CaCO_3$; 10 mg $FeCl_3.6H_2O$; 1 mg each biotin and thiamine, and 0.1 g kanamycin sulfate (km).

DNA isolation and probe preparation

From *E. coli* MV12 cultured in LB broth with 100 μg mL^{-1} Cm, the plasmid pGS9 was extracted by a 25% scale up variation of the procedure described by Maniatis et al. (1982). The ^{32}P DNA probe was made using a nick-translation kit (Bethesda Research Laboratories) and following the suggested protocol. $^{32}PdCTP$ (New England Nuclear) was used as the labeling nucleotide at 3,000 Ci $mmol^{-1}$. The labeled probe was separated from unincorporated nucleotides by using a spun--column procedure (Maniatis et al. 1982).

DNA-DNA hybridization

Streak plates on YMA were prepared after isolation of putative transconjugants to ensure purity of the isolates. These colonies or colonies from soil dilution plates were lifted onto Whatman 541 filters by the procedure of Gergen et al. (1979). The filters were saturated with 6 x SSC (0.15 M NaCl, 0.015 M sodium citrate) and washed for 1 to 2 h in a solution containing 50 mM Tris pH 8, 1 M NaCl, 1 mM EDTA and 0.1% SDS at 37°C with constant agitation to remove cell debris. Salmon sperm carrier DNA and ^{32}P DNA probe were denatured by heating in boiling water for 10 min. and added to heat-sealable bags at concentrations of 150 μg mL^{-1} and 10^5 dpm mL^{-1} hybridization solution, respectively. Bags containing the filters were resealed and incubated at 65°C for 12 to 14 h with agitation. Filters were removed, washed three times in 2 x SSC for 20 min at 65°C, and washed once in 0.2 x SSC at 55°C for 20 min. Autoradiography was carried out at -70°C using XAR-5 X-ray film (Kodak) and processed with a Kodak X-OMAT film processor.

Stability of Tn5 mutants

Six boni fide Tn5 mutants of strain A8 were grown in nonselective YMB for 105 generations and then plated on unamended YMA or YMA containing 300 μg mL^{-1} of Km.

Soil inoculation studies

The soil used was an unnamed silty clay loam Argiborolls collected from Bozeman, Montana, which was passed through a 0.5-mm diameter sieve and homogenized. A moisture retention curve for the soil was determined using a pressure plate for moisture levels from -0.033 to -0.1 MPa and a thermocouple psycrometer for values lower than that range (Richards 1965). Ten grams of soil were added to a series of screw cap flasks (100 cc), brought to either -0.033, -0.3 or -3 MPa, and allowed to equilibrate for ten days. The soil water was then allowed to evaporate, and washed cell suspension from late log phase culture of strain A8T7 was added with enough water to bring the final moisture content to the original levels. The flasks were incubated for 12 hours, each at 15 and 21°C during a 24-hour period. Water content in the soil was determined gravimetrically every other day and water was added if necessary to maintain the moisture potential.

FA counts in the soil

At sampling, 95 mL of extractant solution (Kingsley & Bohlool 1981) was added to each flask together with one drop of Antifoam B emulsion (Sigma Co.) and five drops of Tween 80. The flasks were shaken for 30 min. on a wrist action shaker and the solid particles were pelleted by mild (200 x g) centrifugation. A volume (1 mL) of supernatant was passed through a 0.45 μm-pore size polycarbonate membrane filter (Nuclepore Corp., Pleasanton, CA.) stained with Irgalan Black (Ellis et al. 1984). Fluorescent antibodies to specific chickpea bradyrhizobia were prepared as described by Schmidt et al. (1968) and applied after treatment with rhodamine-gelatin conjugate to eliminate nonspecific absorption of antibody to soil particles (Bohlool & Schmidt 1968). Forty to 70 microscopic fields were counted on each filter and the counts were converted to cells g^{-1} of oven-dry soil. Cells were visualized with a Zeiss microscope equipped for epifluorescence using a HBO Osram mercury light source and a 100X Neofluar objective.

Viable counts

After the above extracting procedure was carried out, but before centrifugation, appropriate dilutions

of the soil suspension were plated on YMA with 200 μg mL⁻¹ Km, 100 μg mL⁻¹ pimaricin and 100 μg mL⁻¹ cyclohexamide, and incubated at 30°C.

Nodule serotyping by FA

Nodule-bearing roots were kept frozen at -20°C until ready for testing. Eighteen nodules were selected per pot, cleaned by shaking for 30 min. in 100 mL water containing 5 drops of Tween 80 and then rinsed in tapwater for 2-3 min. The nodules were individually crushed in 1-2 mL of saline and smears were placed on a glass slide (18 smears/slide). Smears were fixed and stained with the FA conjugate according to the procedure described by Robert & Schmidt (1983).

Competition studies

Strain A8 and five Tn5 mutants of strain A8 were inoculated individually onto pregerminated chickpea seedlings on Leonard jars in the greenhouse. Ninety min. later, strain A15 was inoculated as a challenging strain for each of the above strains. At flowering stage, the nodules were collected and treated as described previously.

RESULTS AND DISCUSSION

Isolation and characterization of Tn5 mutants

Putative mutants, after conjugation with *E. coli* MV-12 were obtained at frequencies from 6×10^{-4} to 1.35×10^{-7} (Table 1). These rates were from 10 to more than 100 fold higher than the spontaneous rates of mutation for the strains in rhizobial minimal salts (RMS) containing 100 μg mL⁻¹ Km. These putative mutants were probed with a ^{32}P (pGS9) probe to confirm the presence of Tn5 inserts. The percentage of mutants that hybridized to the pGS9 probe varied from 9 to 88% (Table 1). Although strain A3 had a frequency of Km resistant cells after conjugation more than 100 fold higher than the spontaneous rate of mutation, only 9% of the transconjugants of that strain hybridized with the pGS9 probe (Table 1). This low rate of boni fide mutants was probably due to instability of the Tn5 insertions that led to loss of the transposon after

the selective pressure was removed during purification of the isolates on YMA. The other three strains tested had a high percent of boni fide mutants (88 to 100%), facilitating the selection of a large number of mutants with Tn5 DNA. A considerable proportion of these Tn5 mutants were resistant to at least 300 to 400 μg mL⁻¹ Km (data not shown). A small percent of the boni fide mutants (less than 13% gave a darker more intensive signal after autoradiography and were resistant to 100 μg mL⁻¹ Cm, suggesting that vector sequences were being maintained.

Some of the A8Tn5 mutants were tested for competitiveness against strain A15 in Leonard jars (Table 2). The percentage of nodule occupancy by these mutants did not differ statistically from the nodule occupancy by the parent strain, even though there was variability in Tn5 mutants (P ≤ 0.05). For example, strain A8T6 containing VI was the least competitive when challenged against strain A15 and the least stable as determined by re-

TABLE 1. Tn5 mutation rate and spontaneous mutation rate for strains of *R. leguminosarum* biovar *phaseoli* and *Bradyrhizobium* sp (Cicer).

Strains[a]	Mutation rate[b]		Boni fide Tn5 Mutants[c]	VI[d]
	Tn5 Transconj.	Spont.		
			%	%
Vik.1	1.45×10^{-7}	0.38×10^{-8}	88	13
Kim.5	2.1×10^{-6}	10^{-7}	NA⁻	NA
A8	2.8×10^{-5}	10^{-7}	100	10
A15	6.1×10^{-5}	10^{-7}	100	0
A16	1.7×10^{-5}	10^{-7}	9	0
A3	6.0×10^{-4}	4.4×10^{-5}	NA	NA

a Strains Vik.1 and Kim.5 are *R. leguminosarum* biovar *phaseoli*. The remaining ones are *Bradyrhizobium* sp (Cicer).

b Rate of mutation in RMS medium amended with 100 μg mL⁻¹ Km.

c Percent of Km⁻ isolates that hybridized to a pGS9 probe.

d Percent of boni fide mutants that show a strong dark signal after hybridization to a pGS9 probe.

e NA, not assayed.

sistance to Km after 105 generations under no antibiotic selection.

Stability of inserted DNA is essential in ecological studies, mainly if the strain is to be used in experiments to be carried out for extended periods. A general stability of Tn5 insertions in *Rhizobium* species was reported by Fredrickson et al. (1988) for strains growing in sterile soil. We tested five boni fide Tn5 mutants and one vector intregrate (VI) transferring them successively on non-selective YMB for 105 generations and plating them on selective (300 μg mL⁻¹ Km) and non-selective medium. For three strains, more than 95% of the bradyrhizobial population was Km resistant (Table 2), confirming the selective (300 μg mL Km) and non-selective medium. For three strains, more than 95% of the brady-rhizobial population was Km resistant (Table 2), confirming the stability of these

TABLE 2. Competitiveness of Tn5 transconjugates of strain A8 and stability of their kanamycin resistant marker.

Strain [a]	Compt.[b]	Plate counts in YMA [c]		Stability [d]
	Nodules with serogroup A8	Km level (μg L⁻¹) 0	300	Population Km resist.
	--- % ---	--- log CFU mL⁻¹ ---		--- % ---
A8	87 (15.3)			
A8T2	84 (14.6)	8.01 (0.07)	8.03 (0.06)	105
A8T3	76 (11.9)	8.01 (0.07)	7.92 (0.06)	81
A8T4	NA	8.10 (0.05)	8.05 (0.02)	89
A8T7	78 (13.0)	8.14 (0.03)	7.98 (0.05)	69
A8T7	78 (14.0)	8.02 (0.09)	8.00 (0.04)	96
A8T8	95 (8.7)	8.31 (0.06)	8.34 (0.04)	107
CV	17%			18%

a A8 is the parent strain; A8T6 is vector integrate (VI) and the remaining are Tn5 inserts.

b Competitiveness in Leonard jars. Each strain was inoculated in pairs with strain A15. Numbers between brackets are standard deviations. The values for the transconjugant strains did not differ from the parent, with the Dunnet's test at p = 95%.

c Total cells after 105 generations in nonselective yeast mannitol broth.

d Strain A8T6 differed statistically from strains A8T2 and A8T8 at p = 95% (LSD = 33.6).

Tn5 inserts, whereas only 69% of the cells of the VI strain remained Km resistant.

Spontaneous antibiotic-resistant mutants have been used extensively in bradyrhizobial ecology (Osa-Afiana & Alexander 1982a, b; Hartel & Alexander 1985, Pena-Cabriales & Alexander 1979), but only recently has transposon-associated antibiotic resistance been used as a selective marker in bradyrhizobial ecology experiments (Fredrickson et al. 1988). The use of Tn5 mutants in ecological studies may be more advantageous than spontaneous mutated strains. Spontaneous mutations usually result in modifications of the antibiotic-sensitive cellular target or cell membranes in a way that reduces their general efficiency (Novick 1980). Consequently, several functions of the cell may be modified, thereby altering the overall environmental competitiveness of the organism, as reported in some previous papers (Jones & Bromfield 1978, Turco et al. 1986, Lewis et al. 1986). In contrast, transposon mutagenesis is usually obtained by a single insertion in the chromosome that codes for an additional protein that enzymatically detoxifies the antibiotic. Therefore, Tn5 mutants are less likely to have impaired biochemical functions as compared to spontaneous mutation. In our studies, the mutation rates of Tn5 transconjugants were much higher than spontaneous mutations (Table 1), and had expressed a high level of resistance to kanamycin (Table 2), that proved to be suitable to ecological studies in a non-sterile soil (Fig. 1). Since the Tn5 transposon also codes for resistance to other antibiotics in *Bradyrhizobium* spp such as streptomycin and neomycin (Selvaraj & Iyer 1983), additional phenotypic markers are available for use, allowing increasing sensitivity for detection of the strain in mixed populations.

Survival of a Tn5 mutant strain under three soil moisture regimes

When the survival of the Tn5 mutant A8T7 was studied in soil at three water potentials in flasks, the highest rate of survival for this

strain was found at -0.3 MPa as shown by the viable count in YMA amended with 200 μg mL^{-1} Km (Fig. 1). At that moisture level, the population of strain A8T7 remained near the level of inoculation for 55 days. In contrast, the plate counts at the two extremes of water potential (-0.03 and -3 MPa) were from one to two orders of magnitude lower than at -0.3 MPa. Previous work (Marshall 1964, Vincent et al. 1962, Rensburg & Strijdom 1980, and Fuhrmann et al. 1986) reported better survival of *Bradyrhizobium* strains at either extremes of dry or wet conditions than at intermediate moisture levels. However, since the energy status of the soil water was not reported, it becomes difficult to extrapolate the results described in these papers. Resistance to desiccation varies among strains of Bradyrhizobium (Rensburg & Strijdom 1980, Fuhrmann et al. 1986), and is affected by the type (Marshall 1964) and amount (Pena-Cabriales & Alexander 1979) of clay in the soil. The lower survival of *Rhizobium* in dry soils in probably due to impairment of vital enzyme functions from the partial loss of cell water (Rensburg & Strijdom 1980), whereas the low survival in moist soils is likely due to antagonism from the soil
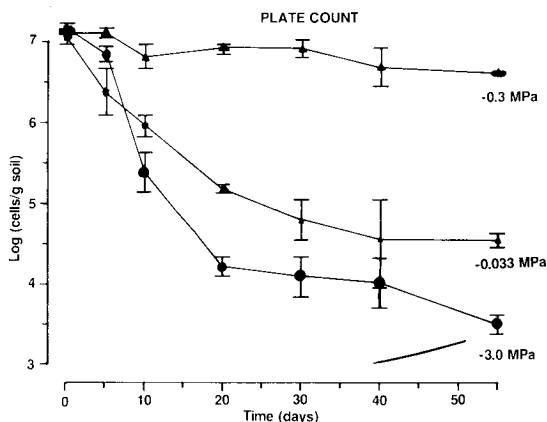
microbial population (Holland & Parker 1966, Schreven 1970). In the present experiment, the moisture level at -0.3 MPa was probably sufficient to reduce the overall soil microbial activity and thereby reduce antagonism, but not dry enough to affect survival of *Bradyrhizobium* sp from soils held a -0.3 MPa.

Enumeration of cells by FA and plate count from soil held at -0.3 MPa were similar throughout the experiment indicating that the entire population of strain A8T7 remained viable at that moisture level (Fig. 2). Additionally, it shows that the Tn5 insert was quite stable in nonsterile soil for at least 55 days, as all the added bradyrhizobial population was recovered by plating dilutions of the soil suspension on YMA with 200 μg mL^{-1} Km.

In contrast, after ten days at -3 MPa, a sharp decline in the viable counts was observed as compared to enumeration by FA (Fig. 3) which suggests that part of the population detected by FA was either dead or no longer culturable, but remained immunologically syable in the soil due the low microbial
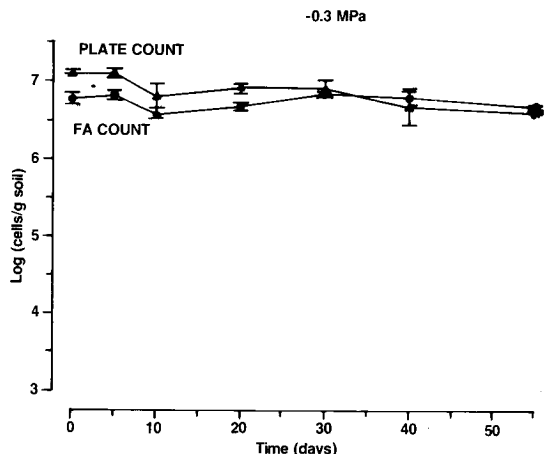

FIG. 1. Effect of soil water potential on survival of the *Bradyrhizobium* sp strain A8T7 as determined by plate counts on YMA containing 200 μg mL^{-1} Km. Vertical bars are standard errors of the mean.

FIG. 2. Enumeration of the *Bradyrhizobium* sp strain A8T7 by plate count on YMA containing 200 μg mL^{-1} Km and by immunofluorescence (FA) from soil maintained at -0.3 MPa water potential. Vertical bars are standard errors of the mean.

activity under the dry conditions. Higher estimates of the bradyrhizobial population in soil by the FA method than by plate count or by MPN reported by Bezdicek & Donaldson (1981) and Kennedy & Wollum (1988) may be due to the inability of the FA technique to .differentiate between dead and alive cells. The discrepancy in enumeration methods may be further explained at low water potentials where cells may be viable but not culturable as suggested for aquatic bacteria (Colwell et al. 1985).

At 30 days, part of the flasks that were maintained at -3 MPa and later wetted to -0.033 MPa, showed an initial increase in the number of the viable cells by plate count (Fig. 3). These results are in agreement with those of Pena-Cabriales & Alexander (1979) who reported growth of *Rhizobium* after wetting a dry soil. Furthermore, viable but non culturable cells held under moisture stress would be expected to increase in plate counts when moisture stress is relieved. In contrast to unaltered treatments, estimates by FA continued to decrease as expected after wetting th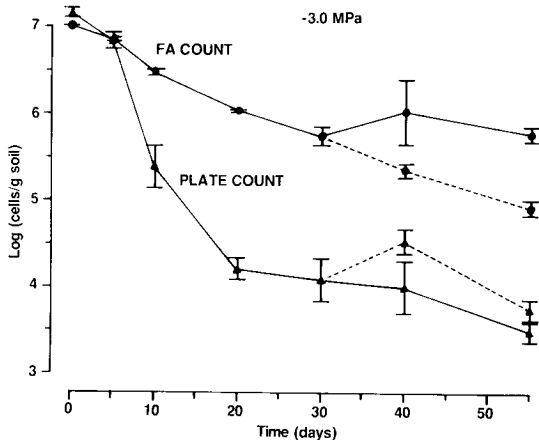e soil, which was probably due to an increase in microbial activity and subsequent decomposition of dead rhizobial cells.

At -0.033 MPa, enumeration by FA and viable count agreed more closely (Fig. 4), which was probably due to a more dynamic microbial activity that would avoid accumulation of stressed cells and dead bradyrhizobial cells. Upon drying at day 30 (dashed line), the viable counts decreased relative to counts from soil maintained at -0.033 MPa which is consistent with the effect of water stress on culturability. Higher estimates by FA after drying of soils held at -0.033 MPa suggest the counting of nonviable cells as discussed before. The soil was submitted to a relatively rapid rate of drying from -0.033 to -3 MPa in less than 24 hours which may be the reason for the initial decline in viable cells. Chao & Alexander (1984) and Mary et al. (1985) observed that fast rates of drying decreased the bacterial survival as compared to slow drying. However, 25 days after starting the drying treatment the population of strain A8T7 was the same at both moisture stress levels for either enumeration method.
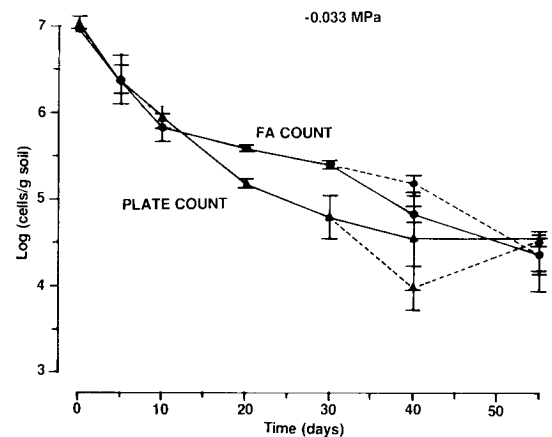

FIG. 3. Enumeration of the *Bradyrhizobium* sp strain A8T7 by plate count and FA from soil maintained at -3 MPa water potential. At 30 days, part of the soil was brought to -0.033 MPa water potential (dashed line). Vertical bars are standard errors of the mean.

FIG. 4. Enumeration of the *Bradyrhizobium* sp strain A8T7 by plate count and FA from soil maintained at -0.033 MPa water potential. At 30 days, part of the soil was brought to 03 MPa water potential (dashed line). Vertical bars are standard errors of the mean.

CONCLUSIONS

1. The results of this study showed that Tn5 mutants of *Bradyrhizobium* sp (Cicer) were good choices for ecological studies.

2. The relative stability of the Tn5 inserts, unaltered competitiveness for most strains and high level of Km resistance expressed in *Rhizobium* sp (Cicer), makes Tn5 mutants probably a better option than spontaneous mutants in those studies.

3. Under nonstressed conditions, both plate count and FA techniques seem to be reliable for enumeration of *Bradyrhizobium* strains. However, under some dry soil conditions, results with these methods show large variations.

4. Enumeration of dead cells by FA and nonculturability of stressed cells by the plate count seems to be the explanation for the discrepancies between these two methods.

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