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THE USE OF *gusA* REPORTER GENE TO MONITOR THE SURVIVAL OF INTRODUCED BACTERIA IN THE SOIL

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ABSTRACT

An effective marker to monitor the survival of introduced bacteria in the soil is required for further evaluation of their beneficial effects on plant growth. This study tested the use of *gusA* gene as a marker to trace the fate of three Gram negative bacteria in the root, rhizosphere, and soil. The study was conducted at the laboratory and greenhouse of the National Institute of Molecular Biology and Biotechnology, Philippines from January to December 2001. Isolates TCaR 61 and TCeRe 60, and *Azotobacter vinelandii* Mac 259 were selected as test bacteria based on their ability to produce indole-3acetic acid and solubilize precipitated phosphate, which may promote plant growth in the field. These bacteria were marked with *gusA* reporter gene from *Escherichia coli* strain S17-1(λ-pir) containing mTn*5*SS*gusA21*. The *gusA* (β-glucuronidase) gene from the donor (*E. coli*) was transferred to each bacterium (recipient) through bacterial conjugation in mating procedures using tryptone-yeast agar followed by the selection of the transconjugants (bacteria receiving *gusA*) in tryptone-yeast agar supplemented with double antibiotics and X-GlcA (5bromo-4chloro-3indoxyl-β-D-glucuronic acid). The antibiotics used were rifampicin and either streptomycin or spectinomycin based on antibiotic profiles of the donor and recipients. The results showed that the insertion of *gusA* gene into bacterial genomes of the recipient did not impair its phenotypic traits; the growth rates of the transconjugants as well as their ability to produce indole-3acetic acid and solubilize precipitated phosphate in pure culture were similar to their wild types. All transconjugants colonized the roots of hot pepper (*Capsicum annuum* L.) and survived in the rhizosphere and soil until the late of vegetative growth stage. The distinct blue staining of transconjugants as the expression of *gusA* gene in media containing X-GlcA coupled with their resistance to rifampicin and streptomycin or spectinomycin made them easier to be recognized and evaluated.

[***Keywords***: *GusA* reporter gene, Gram negative, bacteria, root colonization, rhizosphere, soil]

INTRODUCTION

Monitoring the survival of bacteria introduced into non-sterile soil is important to ensure their beneficial effects on plant growth. Glick (1995) recommended tracing the introduced bacteria in natural environment since no guarantee that good bacteria based on laboratory screening will have a significant impact on plants in the field. This implies that some introduced bacteria may not be able to compete with native microorganisms to sustain their population in the soil. Certain bacteria that have ability to proliferate and persist longer than other microorganisms will provide them with a competitive advantage over others and be considered as good candidates for microbial inoculant (Kennedy 1998). To investigate this ability and provide a better understanding of plant-microbe interaction, an effective marker is required.

Several methods in marking bacteria have been developed from traditional to modern methods. The traditional methods such as antibiotic resistant markers are considered as a laborious and expensive work. In some cases, developing bacteria carrying antibiotic resistance on highly selective media may alter some phenotypic characteristics, which may not be readily detectable (Hagedorn 1994; Morgan and Winstanley 1997). On the other hand, the modern methods such as marker genes are simpler and involve observation of colors by unaided eye. These methods also permit the quantitative assay and spatial localization of marked bacteria in the root and soil environment (Wilson *et al*. 1995; Subba-Rao 1999).

A number of reporter genes used as marker for Gram negative bacteria have been developed. It includes *gusA* gene encoding β-glucuronidase, *lacZ* gene encoding β-galactosidase, *xylE* gene encoding catechol 2,3-dioxygenase, and different sets of luciferase genes. The *gusA* gene is the most widely used reporter gene in plant molecular biology because of the absence of *gus* activity in agronomically important bacteria such as *Rhizobium*, *Bradyrhizobium*, *Pseudomonas*, *Agrobacterium*, and *Streptomyces* and in plant they might associate (Wilson 1995; Wilson *et al*. 1995; Wilson 1996). To date, use of *gusA* gene as a marker has been applied intensively for rhizobial competition studies, but its application for Gram negative bacteria other than *Rhizobium* is still rare.

The important characteristic of *gusA* gene as a marker is that it encodes β-glucuronidase which cleaves the histochemical substrates such as X-GlcA (5bromo-4chloro-3indoxyl-β-D-glucuronic acid). The cleavage product of the substrate gives rise to indigo precipitate (Wilson 1995). Thus, bacteria marked with this *gusA* gene can easily be detected as blue colonies on plates or blue areas on the root surface or inside root tissue (Wilson 1996).

This study tested the use of *gusA* reporter gene in marking three Gram negative bacteria to monitor their survival in the root, rhizosphere, and soil. It includes the characterization of transconjugants (bacteria receiving *gusA* gene) and their wild types to ensure no major change in their phenotypic traits due to the insertion of *gusA* gene. The result of this study will provide an advanced technology in marking bacteria for the selection of highly competitive inoculant straints or other practical uses, which is far simpler than previous methods.

MATERIALS AND METHODS

This study was conducted in the laboratory and greenhouse of the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines at Los Banos, Philippines from January to December 2001. All bacteria (the recipients and donor of *gusA* gene) were provided by BIOTECH.

Bacteria and Donor Strains

Three Gram negative bacteria, isolates TCaR 61 and TCeRe 60, and *Azotobacter vinelandii* Mac 259, were used in the experiments as the recipient of *gusA* gene. Isolates TCaR 61 and TCeRe 60 were isolated from rhizosphere of *Calopogonium* sp. and root extract of *Centrosema pubescens,* respectively in Tarlac areas, Philippines. The selection of these bacteria is based on their ability to produce indole-3acetic acid (IAA) (Paterno 1997) and solubilize precipitated phosphates (Husen 2003), which may promote plant growth in the field by increasing root hairs and P uptake although their effects on the growth of hot pepper (*Capsicum annuum* L.) in greenhouse experiment were not significantly different from uninoculated control (Husen and Saraswati 2003).

The donor of *gusA* reporter gene was from *Escherichia coli* strain S17-1 (λ-pir) that harbored plasmid pCAM121 containing mTn*5*SS*gusA21*. Besides encoding enzyme β-glucuronidase, this GUS transposon (mTn*5*SS*gusA21*) also encodes streptomycin, spectinomycin, and ampicillin resistance (Wilson *et al.* 1995; Wilson 1996), which is important in marking procedures and recovering the transconjugants from rhizosphere and soil.

Antibiotic resistance of each recipient and the donor (*E. coli*) was determined by their ability to grow in Tryptone-Yeast (TY) agar (containing 5 g tryptone, 3 g yeast extract, 0.87 g $CaCl_2.H_2O$, and 12 g agar in 1000 ml distilled water) supplemented with different concentration of ampicillin, streptomycin, spectinomycin, and rifampicin. The donor was able to grow in TY agar containing 100 ppm streptomycin or 100 ppm spectinomycin, but not in 5 ppm rifampicin. On the other hand, the recipients grew well in 5 ppm rifampicin, but not in 100 ppm streptomycin or 100 ppm spectinomycin. Therefore, TY agar containing 5 ppm rifampicin and either 100 ppm streptomycin or 100 ppm spectinomycin (selective media) that suppress the growth of both the donor and recipient were used to select the transconjugants (bacteria receiving *gusA* gene).

Marking Procedures

Insertion of *gusA* gene from *E. coli* (donor) to each recipient through bacterial conjugation was conducted by mating of each recipient with the donor according to the procedure described by Wilson (1996). Each recipient and donor was grown in TY broth overnight. Each culture (200 µl) was centrifuged at 6000 rpm for 5 minutes in sterile Eppendorf tube. The cell pellet was washed several times with 0.8% NaCl and then re-suspended in 0.8% NaCl. The cell pellets of recipient and donor were mixed and about 50 µl of the mixture was inoculated to TY agar without antibiotic (non-selective media). After overnight incubation, the growth was scraped and streaked onto several plates containing selective media (TY agar containing 5 ppm rifampicin and 100 ppm streptomycin or 100 ppm spectinomycin). At the following day, the isolated colonies were streaked again onto selective media + 50 µg ml^{-1} X-GlcA. Blue colonies of transconjugant as the expression of *gusA* gene were observed after 24-72 hours incubation.

Characterization of Transconjugants and Wild Types

Assessment of the main detectable characteristics of each transconjugant and its wild type was conducted to ensure no major changes in phenotype due to the insertion of GUS gene into the recipient's genome as suggested by Wilson (1996). It consisted of growth rate, IAA production, and P solubilization.

The growth rate to be compared was conducted by enumerating the number of cell increase in the period

of 7 days. Media used was IAA-production medium (minimal salt medium + L-tryptophan) as described by Frankenberger and Poth (1988). Each sample (transconjugant and wild type) was grown in TY broth supplemented with appropriate antibiotics (rifampicin and streptomycin/spectinomycin for transconjugants and rifampicin for wild types). After 24 hours, 0.5 ml of the culture was transferred to 50 ml IAA-production medium. This medium also contained the appropriate antibiotics for the growth of each sample. The number of cells was determined by Miles and Misra drop plate method (Miles and Misra 1938) at 0 and every 30 minutes for the first 3 hours, every 1-2 hours until 12 hours, every 12 hours until 36 hours, and every 24 hours until 168 hours (7 days).

The ability of transconjugant and wild type to produce IAA was assayed colorimetrically using ferric chloride-perchloric acid reagent ($FeCl_3$-$HClO_4$) according to procedures of Gordon and Weber (1951). About 1.5 ml of each 24-hour IAA-production medium was centrifuged at 12,000 rpm for 5 minutes to separate the cells with supernatant. One milliliter of supernatant was added to 2 ml $FeCl_3$-$HClO_4$ reagent. The development of color (yellow to red) as an indicator of the presence of IAA in the medium was used to compare the IAA produced by each transconjugant and its wild type.

P solubilization test was conducted using Pikovskaya's agar containing 5 g $Ca_3(PO_4)_2$ per liter (Subba-Rao 1999). The transconjugant and its wild type from 24-hour culture were streaked on the surface of replicated Pikovskaya's agar plates. The presence of clearing zone around the bacterial growth as the indication of P solubilization activity was noted after overnight incubation.

Inoculation, Growing of Seedlings, and Transplanting

Prior to inoculation, hot pepper seeds, similar in size, were sterilized by soaking in 1% sodium hypochlorite for 10 minutes. Seed inoculation was performed by soaking the seeds in broth culture of transconjugant for 2 hours. The inoculated and uninoculated seeds were sown in trays containing sterilized sand for 32 days. Soil for growing the seedlings was prepared in polyethylene bags containing 3 kg air-dried soil. The soil received N, P_2O_5, and K_2O from ammonium phosphate and potassium chloride (0.75 and 0.29 g bag^{-1} of ammonium phosphate and potassium chloride, respectively). At transplanting, the healthy seedlings were soaked again in broth culture for 2 hours before transplanting. One milliliter of broth culture containing approximately 10^8 cells ml^{-1} of TCaR 61, 10^7 cells ml^{-1} of TCeRe 60, and 10^8 cells ml^{-1} of Mac 259 was applied to each seedling. The seedlings were grown in the soil for 26 days.

Root Colonization

Colonization of the roots by transconjugants was monitored periodically at 15, 21, and 31 days after seed inoculation, and at 10 and 26 days after seedling inoculation. Harvested roots were rinsed several times in saline solution (0.85% NaCl) to remove any adhering sand or soil. Cleaned roots were immersed in saline solution containing 500 µg ml^{-1} of X-GlcA and incubated at 37°C until 24 hours. After good staining observed, the roots were rinsed in fresh saline without X-GlcA followed by soaking in 2.5% sodium hypochlorite for 30 minutes to clear the tissue. The extent by which the transconjugants colonize the roots was observed under a photomicroscope.

Recovery of Transconjugants from Soil and Rhizosphere

The survival of the transconjugants in the soil and rhizosphere was monitored at four sampling times (10, 12, 18, and 26 days after seedling inoculation). These were conducted using plating assays and colony forming unit counts to determine the number of transconjugants per gram of dry soil (Zuberer 1994). The medium to grow and enumerate the transconjugants was TY agar + double antibiotics (5 ppm rifampicin, 100 ppm streptomycin or 100 ppm spectinomycin) + 1.25 ppm anti-fungal Brilliant Green + 50 µg ml^{-1} X-GlcA.

Soil samples were collected at four quadrants of 4 cm distance from the base of the plant. The volume of soil sample per quadrant collected from the surface of soil was approximately 16 cm^3 (2 cm length, 2 cm width, and 4 cm depth). Ten grams of moist soil sample were diluted with 95 ml sterile saline solution. After shaking the soil suspension by hand for 50 times, a series of two further 10-fold dilutions was made by transferring 1 ml soil suspension to 9 ml diluents. Each dilution (100 µl of aliquots) was spread on the media with two replications per dilution. The blue colonies of the transconjugants were observed after 2-4 days after incubation at room temperature. To determine the numbers of transconjugant cells recovered per gram dry-weight soil, soil moisture content of 10 g of separate moist soil sample was determined after 24 hours drying at 105°C.

Rhizosphere soil samples were collected from soil adhering to root (after the loose soil and clumps have been removed). The root-soil was placed in 100 ml dilution bottle containing 25 ml saline solution and

shaken using rotary shaker at 150-200 rpm for 10 minutes. This soil solution (stock solution) was diluted to make a series of three 10-fold dilutions. In a similar way to the recovery of the transconjugants from soil, 100 µl of aliquots of each of the three dilutions was spread on media with two replications per dilution and incubated for 2-4 days. To determine the numbers of transconjugant cells recovered per gram dry-weight of rhizosphere soil, separate 10 ml of stock soil suspension was air dried for 3-5 days followed by overnight drying at 105ºC.

RESULTS AND DISCUSSION

The success of each bacterium receiving *gusA* gene was indicated by its ability to grow in media containing double antibiotics (5 ppm rifampicin and 100 ppm streptomycin or 100 ppm spectinomycin) that prevented the growth of the donor (*E. coli*) and recipient (the wild type). The blue colonies of transconjugants in the media containing 50 µg ml^{-1} X-GlcA (5bromo-4chloro-3indoxyl-β-D-glucuronic acid) designated the activity of GUS gene in the cleavage of this histochemical substrate that yielded indigo precipitate. The addition of Brilliant Green (1.25 ppm) to prevent the growth of fungus did not affect the growth of transconjugants and their blue colonies were still distinct from light green background of the media.

Growth Rate, IAA Production, and P Solubilization

The comparison of growth rate pattern between transconjugants and their wild types in pure culture is shown in Fig. 1. Transconjugants TCaR 61 and Mac 259 showed similar growth pattern with their wild types and reached their late exponential phase at around 48 and 48-76 hours after incubation, respectively. Transconjugant TCeRe 60, on the other hand, showed slower growth rate than its wild type and required more than 76 hours to reach its late exponential phase. The overall results, however, indicated no significantly different growth rate between transconjugant and its wild type as caused by the insertion of *gusA* gene.

The ability of the transconjugants to produce IAA and solubilize precipitated calcium phosphate in pure culture was also similar to their wild types. These results confirmed that the insertion of *gusA* gene from the donor into the recipient genomes did not impair these beneficial traits.

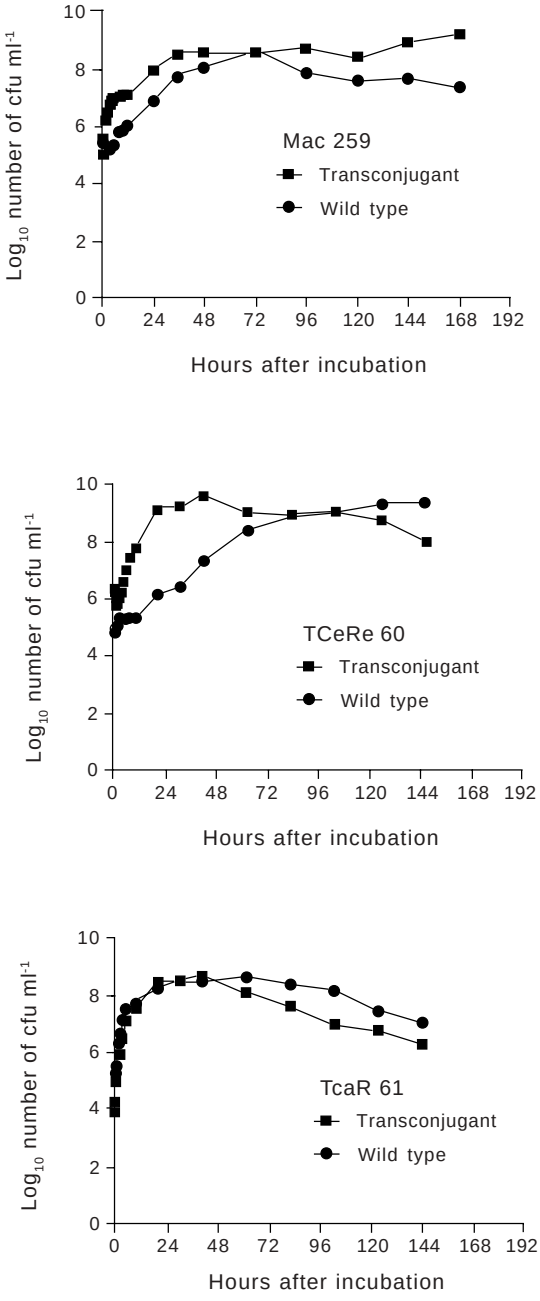

Fig. 1. Growth rate patterns of transconjugants vs wild types of three Gram negative bacteria TCaR 61, TCeRe 60, and *Azotobacter vinelandii* Mac 259

Root Colonization

The spatial and temporal root colonization by transconjugants was exhibited by discrete region of blue staining on the roots (Fig. 2). At seedling stages, all inoculated roots were colonized by transconjugants. The degree of colonization, however, decreased from young to old seedlings as indicated by light blue color on the roots at 31 days after seed inoculation. This

implies that reinoculation of plant at transplanting is necessary to sustain the number of bacterial population in the root. On the other hand, the decrease of root colonization after transplanting seemed to be lower than that of seedling stages since the quality of blue staining of the root was more or less the same until the end of experiment. Chabot *et al*. (1996) emphasized the importance of root colonization by introduced bacterial cells to suppress the growth of deleterious and pathogenic organisms. The overall results showed the ability of these three introduced bacteria to colonize the root until the end of the experiment. The results also indicated that root exudates, which function as a selector of different organisms in the rhizosphere (Rovira 1965; Grayston *et al*. 1998) released by hot pepper were favorable for growth and development of transconjugants although the main factors that influence root colonization are still not known (Elliot-Juhnke *et al*. 1987).

Population of Transconjugants in the Rhizosphere and Soil

Plating assays to count the transconjugants recovered from the rhizosphere and soil showed that the transconjugants as well as other native soil bacteria that were resistant to rifampicin, streptomycin, and spectinomycin grew together in the plates after 2-4 days of incubation. The blue colonies of transconjugants were in contrast to the white and yellow colonies of native soil bacteria (Fig. 3). The distinct blue colonies of these GUS marked bacteria and differentiated them with native soil bacteria that were also resistant to antibiotics made them easier to be recognized. This justified that marking bacteria with *gusA* gene is an efficient and powerful tool in monitoring the fate of introduced bacteria in the soil.

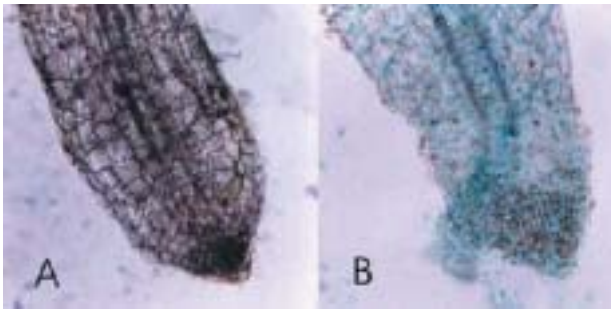

Fig. 2. Photomicrographs (100x) of hot pepper root tips at 31 days after seed inoculation showing no colonization (A, control) and colonization (blue color) of transconjugant TCeRe 60 (B)

The number of transconjugants recovered from rhizosphere and soil is shown in Fig. 4 and 5. It shows the changes in the number of colony forming unit (cfu) at four sampling times after soil inoculation with 10^7-10^8 cells ml^{-1} of transconjugant broth culture.

Ten days after seedling inoculation or 10 days after transplanting (DAT), the number of transconjugants recovered from the rhizosphere soil was 6.1-6.5 $\log_{10}$ cfu g^{-1} oven-dry soil or higher than those from the soil (4.0-4.4 $\log_{10}$ cfu g^{-1} oven-dry soil). This explains the increase of nutrient levels in the rhizosphere soil compared with soil as reported by Thompson *et al*. (1992). In the rhizosphere soil, the number of transconjugants increased at 12 DAT and decreased afterwards. At the end of the experiment (26 DAT), transconjugants Mac 259 and TCeRe 60 attained a population of about 4.0-4.5 $\log_{10}$ cfu g^{-1} oven-dry soil, while transconjugants TCaR 61 had a population of 6 $\log_{10}$ cfu g^{-1} oven-dry soil. In the soil, there was a sharp decrease in transconjugant populations at 10 DAT followed by a slight increase of transconjugants Mac 259 and TCeRe 60 and a slight decrease of transconjugants TCaR 61 at 18 DAT. Subsequently, the population decreased to about 3.0-3.2 $\log_{10}$ cfu g^{-1} oven-dry soil at 26 DAT.

The decreasing trend of bacteria population in the rhizosphere and soil as the plant started mature is mainly due to the decrease of root exudates (Rovira 1965). However, the variable changes of each transconjugant population could be resulted from their different ability to cope with the daily fluctuation of soil moisture, the decrease of root excretion, and microbial antagonism as describe by Vandenhove *et al*. (1991). The overall results showed the ability of transconjugants to establish and survive in the soil

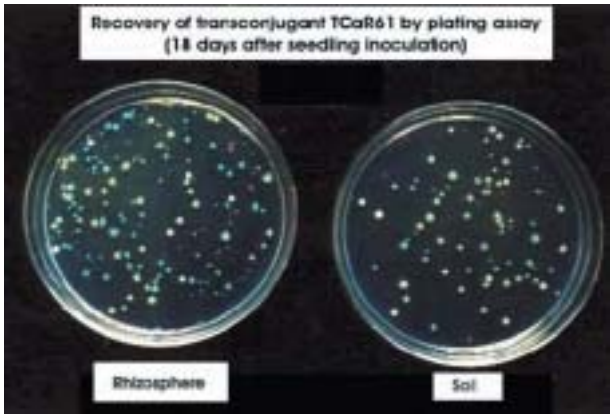

Fig. 3. Blue colonies of transconjugant TCaR 61 in Tryptone-Yeast agar supplemented with X-GlcA, rifampicin and spectinomycin, and Brilliant Green, which are in contrast to white or yellow colonies of other bacteria recovered from rhizosphere and soil.

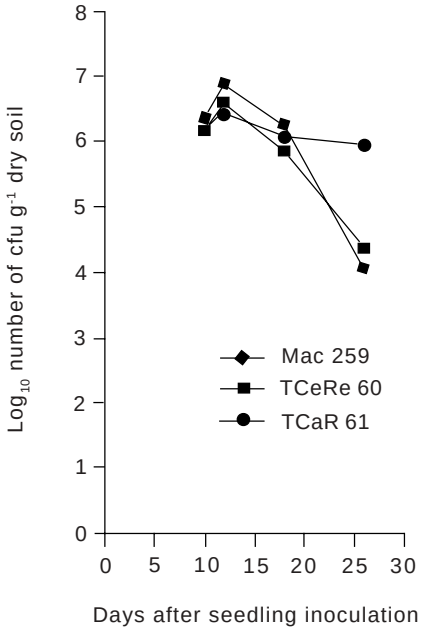

Fig. 4. Survival of transconjugants of three Gram negative bacteria TCaR 61, TCeRe 60, and *Azotobacter vinelandii* Mac 259 in the rhizosphere.

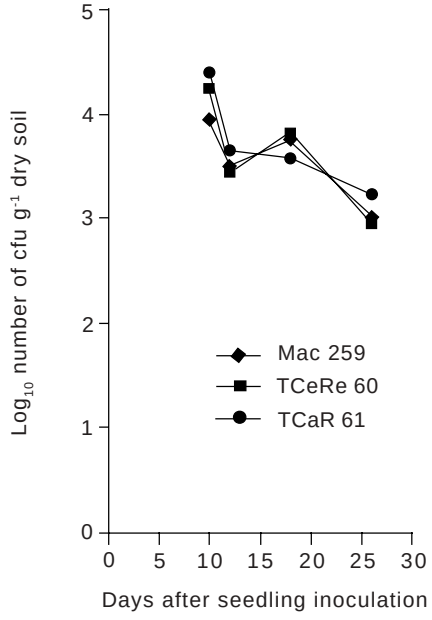

Fig. 5. Survival of transconjugants of three Gram negative bacteria TCaR 61, TCeRe 60, and *Azotobacter vinelandii* Mac 259 in the soil.

since their population were still detected at the late growth of vegetative stage (26 DAT). Transconjugant TCaR 61, however, was much more survive or competitive than TCeRe 60 and Mac 259 (Fig. 4). Thus, any effect of these three bacteria on the growth of hot pepper as reported by Husen and Saraswati (2003) is not related directly to their survival in the root, rhizosphere, and soil.

CONCLUSION

Marking bacteria with *gusA* reporter gene to monitor their survival in the soil has been demonstrated by simple mating procedures and worked with three Gram negative bacteria; isolates TCaR 61 and TCeRe 60, and *Azotobacter vinelandii* Mac 259. The insertion of *gusA* gene from the donor of *Escherichia coli* strain S17-1 (λ-pir) into bacterial genomes of each recipient did not impair its phenotypic traits; the growth rates of these three GUS-marked bacteria (transconjugants) and their ability to produce indole-3acetic acid and solubilize precipitated phosphate in pure culture were similar to their wild types. All transconjugants colonized the roots of hot pepper and survived in the rhizosphere and soil until the late of vegetative stage. Blue staining of the roots as the presence of the transconjugants on the surface and inside the root as well as their blue colonies on media containing X-GlcA (5bromo-4chloro-3indoxyl-β-D-glucuronic acid) that were in contrast to white and yellow colonies of native soil bacteria made them easier to be recognized and evaluated. However, validation of this promising method for various practical situations and uses is recommended to ensure its reability and safety.

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