



# Enhanced survival of *Pseudomonas fluorescens* in soil following establishment of inoculum in a sterile soil carrier

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## Abstract

The method by which bacterial inocula are added to soil can greatly affect survival. This study assessed the establishment of bacteria in a carrier soil, prior to inoculation, as a method of enhancing survival in an agricultural soil. Preincubation of *Pseudomonas fluorescens* in a sterile soil carrier led to greater survival in soil microcosms than preincubation in a non-sterile soil carrier or inoculation of a liquid cell suspension. Increased preincubation time in the sterile soil carrier resulted in greater survival after inoculation. *P. fluorescens* colonised in a sterile soil carrier for 2 weeks declined in concentration after inoculation by only 2 and 4 log units after 1 and 2 months, respectively, whereas bacteria inoculated using a cell suspension were undetectable by 1 month. Activity of *P. fluorescens* in soil microcosms, as determined by luminescence, decreased rapidly in all inoculation treatments to undetectable levels by day 4. Results show that establishment and growth using nutrients in sterile soil may adapt cells to soil environmental conditions, thereby increasing survival of bacterial inocula. © 2000 Elsevier Science Ltd. All rights reserved.

**Keywords:** Bioluminescence; Inoculum; *Pseudomonas fluorescens*; Survival; Soil

## 1. Introduction

Microbial inoculation of soil is required for a number of applications, such as plant growth promotion, inhibition of plant pathogens, biodegradation of toxic compounds, soil structure improvement and microbial leaching of metals (van Veen et al., 1997). The maintenance of cell survival and activity in both rhizosphere and non-rhizosphere soils is an important consideration for the success of any inoculation protocol. Introduction of bacteria into the plant rhizosphere is often successful (Punja, 1997), as conditions for microbial survival and growth are favourable. However, the failure of biocontrol agents to prevent seedling or

root rot diseases of plants, as well as infections during the later stages of plant growth, may be due to poor bacterial survival in soil (Miller et al., 1990; van Veen et al., 1997). Non-rhizosphere soil can be unfavourable for microbial growth, and introduction of bacterial suspensions often results in rapid decreases in population size and activity (van Veen et al., 1997). To facilitate introduction of high cell numbers and increase survival of micro-organisms in soil, inoculum formulations using carrier materials have been used.

Carrier materials may act to enhance survival of inocula by providing micro-organisms with a protective environment in order to escape unfavourable conditions in soil. The reasons for a decrease in microbial inoculum populations in soil over time include insufficient nutrients available for maintenance and replication, and suboptimal environmental conditions, such as matric potential, pH, ionic strength and temperature (van Elsas and van Overbeek, 1993). Predation by bacterivorous micro-organisms, such as protozoa, and

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competition with indigenous bacteria can also decrease inoculum concentrations. To be successful, a carrier material must enhance survival of inocula during storage and after introduction into soil. Ease of handling and application of the formulation to the target site, cost of materials and production, and toxicity are also important considerations.

Most carrier materials and protocols have been studied for use as legume inoculants. The simplest bacterial preparation is a liquid suspension, but problems with handling and poor survival during storage and after addition to plant seed and soil have led to the use of alternative methods. Freeze-dried preparations or addition of cells to talc or carboxymethylcellulose have been used, but many bacteria do not survive well using these dry formulations. The most widely used carriers for legume inoculants are natural compounds, such as peat, soil, compost and plant materials (Thompson, 1980; van Veen et al., 1997). Peat has been shown to improve inoculum effectiveness over non-peat formulations (Roughly, 1970; Walter and Pau, 1993), but problems due to variability in composition of peat based materials (Trevors et al., 1992) and possible production of toxic products during sterilisation (Roughly and Vincent, 1967; Strijdom and van Rensburg, 1981) can affect performance. Clay is another natural substance that enhances microbial survival, although large amounts of added clay can affect the physical properties of soil (Heijnen, 1992). Although these natural materials have been commonly used as inoculum carriers, there is a lack of quantitative survival studies and little information regarding non-rhizosphere applications.

Encapsulation of bacteria in defined polymers, such as agarose, alginate,  $\kappa$ -carrageenan or polyurethane, has also been investigated, and is effective for long-term storage and enhanced survival in agricultural soil (Leung et al., 1995; Trevors et al., 1993) and pentachlorophenol (PCP) contaminated soils (Briglia et al., 1990; Cassidy et al., 1997). Although effective, the cost in terms of materials and production may limit applications using these polymeric carrier materials as compared to more easily prepared formulations. Also, it may be undesirable to introduce even non-toxic foreign material into the soil environment, as the effect on soil populations or dynamics may be difficult to assess.

The aim of this study was to use *Pseudomonas fluorescens* to test the effect of introducing bacteria into sterile soil before inoculation into a non-plant agricultural soil environment. *P. fluorescens* MON787 used in this study was genetically modified with rifampicin resistance and *luxAB* genes to allow selective recovery and assessment of metabolic activity in environmental samples, and was previously used to monitor bacterial recovery of activity following starvation in soil (Van Dyke and Prosser, 1998). It was hypothesised that the

establishment of cells in soil prior to inoculation may enhance survival due to increased resistance to environmental stress.

## 2. Materials and methods

### 2.1. Soil characteristics

An Inch sandy loam soil was used; organic C, 2.13%; organic N, 0.19%. The soil was 3-mm sieved, and the pH adjusted with  $\text{Ca}(\text{OH})_2$  to 6.7 in 0.01 M  $\text{CaCl}_2$ . Sterile soil was prepared as above, followed by autoclaving for 1 h on each of three successive days.

### 2.2. Bacterial strain and inoculum preparation

*P. fluorescens* MON787, chromosomally marked with rifampicin resistance and *luxAB* genes from *Vibrio harveyi*, was previously described by Van Dyke and Prosser (1998). *P. fluorescens* was grown in Luria broth to late log phase, harvested by centrifugation at  $8000 \times g$  and  $4^\circ\text{C}$  for 10 min, and washed twice in equal volumes of 0.01 M phosphate buffered saline (PBS), pH 7.0. Cells were resuspended in an equal volume of PBS, incubated at  $25^\circ\text{C}$  for 2 h, and collected by centrifugation as described above. Cells were resuspended in sterile distilled water before addition to soil.

Colonised carrier soils for addition to soil microcosms were prepared by addition of cells in either sterile or non-sterile soil. Soils with an initial matric potential of  $-13$  MPa were added to 250 ml sterile Duran bottles and inoculated with *P. fluorescens* to give final cell concentrations of  $1 \times 10^9$  or  $1 \times 10^8$  cfu  $\text{g}^{-1}$  dry wt soil and a matric potential of  $-30$  kPa.

Colonised carrier material prepared using sterile soil was incubated for 0, 7 or 14 days in the dark at  $25^\circ\text{C}$  before addition to microcosms. Colonised non-sterile soil was added directly into soil microcosms.

### 2.3. Microcosm design

Soil microcosms consisted of 250 ml polypropylene beakers containing 50 g (dry wt) soil at an initial matric potential of  $-13$  MPa. Colonised sterile or non-sterile carrier soil, prepared as described above at  $1 \times 10^9$  or  $1 \times 10^8$  cfu  $\text{g}^{-1}$  soil, were added to non-sterile soil microcosms at 1 or 10% (w/w), resulting in a final concentration of  $1 \times 10^7$  cfu  $\text{g}^{-1}$  soil. Control microcosms were prepared by adding a suspension of *P. fluorescens* in water to non-sterile soil or non-sterile soil premixed with 1 or 10% (w/w) sterile soil, resulting in a final cell density of  $1 \times 10^7$  cfu  $\text{g}^{-1}$  soil. The final soil matric potential was adjusted using bacterial inocula and water to  $-30$  kPa. Soil was thoroughly

mixed with a spatula, and beakers were covered with foil. Microcosms were incubated in the dark at 25°C and maintained at -30 kPa.

#### 2.4. Bacterial enumeration

Bacterial cell concentrations were monitored in all microcosms at intervals by removal of 1 g (dry wt) soil from each microcosm. Soil samples were placed in sterile glass Universal bottles, diluted in 10 ml PBS and shaken at high speed for 20 min on a vertical shaker (Stuart Scientific, UK). Samples were serially diluted in PBS and plated in triplicate onto Luria agar containing 50 µg rifampicin and 50 µg cycloheximide ml<sup>-1</sup>. Colonies were counted after incubation of plates for 24 and 48 h at 30°C.

#### 2.5. Luminescence measurements

Luminescence was measured as described by Van Dyke and Prosser (1998). Briefly, a 1.2-ml sample was

removed from the 10% soil dilution prepared for bacterial enumeration and centrifuged at 500 × g for 1 min. One ml of supernatant was added to a luminometer cuvette with 10 µl of 10% (v/v) *n*-decanal in ethanol, vortexed briefly, and incubated at 25°C for 5 min. Luminescence was monitored at 25°C using a BioOrbit 1252 luminometer equipped with MultiUse software (Bio-Orbit, Turku, Finland). Values were calculated as the light output integrated over 10 s of incubation, and expressed as relative light units (rlu). Triplicate measurements were taken for each sample.

#### 2.6. Statistical analysis

All microcosm studies were performed in triplicate. Treatments at each time point were compared by analysis of variance performed on logarithmically transformed data using Minitab V.11, with significance between treatments determined using Tukey's multiple comparison test ( $P = 0.05$ ). Variability is indicated in the figures as error bars representing the standard deviation between three replicate samples.

### 3. Results

#### 3.1. Effect of inoculation method on bacterial survival in soil microcosms

*P. fluorescens* inoculated directly into non-sterile soil as a cell suspension decreased rapidly from an initial concentration of  $1 \times 10^7$  to  $9 \times 10^5$  cfu g<sup>-1</sup> soil after 2 days of incubation. Cell numbers then continued to decrease, and were below the detection level of  $1 \times 10^2$  cfu g<sup>-1</sup> at day 28 (Fig. 1A and B). A liquid cell suspension of *P. fluorescens* was also inoculated directly into non-sterile soil premixed with 1% sterile soil to determine whether the nutrients released during autoclaving would affect cell viability, but the pattern of cell decline was the same as in non-sterile soil without sterile soil additions (Fig. 1A). In a further control, bacteria were first added to a non-sterile soil carrier at a concentration of  $1 \times 10^9$  cfu g<sup>-1</sup> soil, and added without preincubation at 1% (w/w) into non-sterile soil. Cells in the non-sterile soil carrier were not preincubated as the rapid decrease in viability would result in low initial inoculum concentrations, thus preventing comparison with other treatments. Initially, the bacteria in this control followed a pattern similar to that of free cells added to non-sterile soil, but values were significantly higher than the free cell control at day 21 and were still detectable at day 28.

*P. fluorescens* was also introduced into soil microcosms using a sterile soil carrier, following preincubation for 0, 7 and 14 days. In all cases, this led to survival at higher levels than for cells added using a

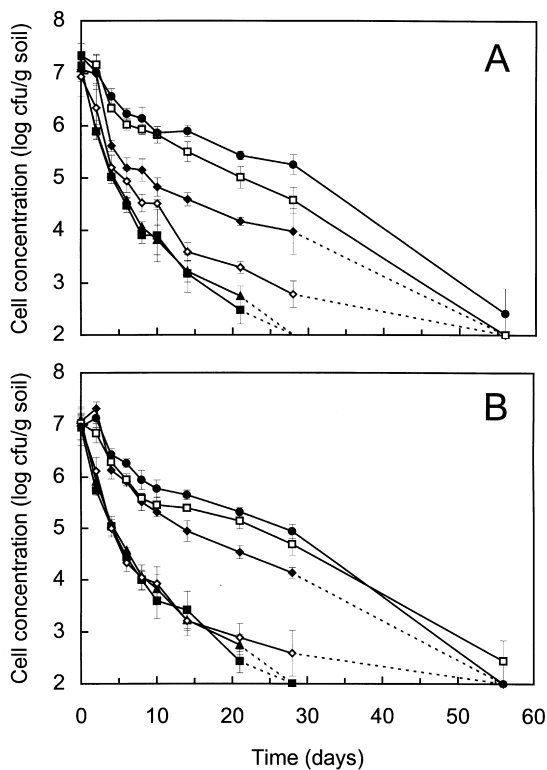


Fig. 1. Effect of inoculation treatment on survival of *P. fluorescens* MON787 in soil. *P. fluorescens* was preincubated in sterile soil for 0 (◆), 7 (□) or 14 (●) days at  $1 \times 10^9$  (A) or  $1 \times 10^8$  (B) cfu g<sup>-1</sup> before addition at 1% (A) or 10% (B) (w/w) to non-sterile soil microcosms. Control treatments include addition of *P. fluorescens* in a non-sterile soil carrier (◇) and addition of a liquid cell suspension directly to non-sterile soil (▲) or to non-sterile soil premixed with uninoculated sterile soil (■). The detection limit was  $1 \times 10^2$  cfu g<sup>-1</sup> and broken lines indicate values below the detection limit. Error bars represent the standard deviation between three replicate samples.

liquid carrier, and the length of the preincubation period in sterile soil determined the survival patterns. The viability of *P. fluorescens* preincubated in a sterile soil carrier for 7 or 14 days remained significantly higher than control values throughout the experiment, and could still be detected at day 56 when all other treatments were below the detection limit (Fig. 1A). The viability of cells added in a sterile soil carrier but not preincubated dropped rapidly to control levels at day 4, but after 14 days this treatment resulted in significantly higher cell counts than control treatments (Fig. 1A). Overall survival of *P. fluorescens* preincubated in a sterile soil carrier was higher than cells added in a sterile soil but not preincubated. At days 14, 21 and 28, survival was higher with increased preincubation times in the sterile soil carrier.

The above experiment was repeated using inoculum incubated in a sterile soil carrier at the lower concentration of  $1 \times 10^8$  cfu  $g^{-1}$  for 0, 7 or 14 days. Colonised sterile soil carriers were added at 10% (w/w) to non-sterile soil microcosms, to give the same final cell concentration of  $1 \times 10^7$  cfu  $g^{-1}$ . Control microcosms were prepared as described above, except that 10% sterile soil or colonised non-sterile soil was added as required. The overall survival patterns were similar to those observed for 1% sterile soil inoculum additions. Control treatments of *P. fluorescens* added as cell sus-

pensions were undetectable at day 28, and cells added in a non-sterile soil carrier decreased to  $5 \times 10^2$  cfu  $g^{-1}$  at day 28 (Fig. 1B). *P. fluorescens* added to non-sterile soil microcosms in a sterile soil carrier again showed higher cell viability than control treatments. Preincubation in sterile soil for 7 or 14 days resulted in survival of  $5 \times 10^4$  and  $9 \times 10^4$  cfu  $g^{-1}$  at day 28, respectively, and cells could still be detected at concentrations of  $4 \times 10^2$  and  $1 \times 10^2$  cfu  $g^{-1}$  at day 56 (Fig. 1B). There were no significant differences between treatments preincubated in sterile soil for 7 or 14 days. Cells in a sterile carrier soil and added immediately to soil microcosms decreased from  $1 \times 10^7$  to  $1 \times 10^4$  cfu  $g^{-1}$  at day 28, and were undetectable by day 56 (Fig. 1B). From day 14 to day 56, treatments added in a sterile carrier soil without preincubation were significantly less than those which had been preincubated for 7 or 14 days.

### 3.2. Bacterial activity in soil microcosms

In addition to cell concentration (Fig. 1A and B), the activity of *P. fluorescens* in soil was determined by luminometry. Microcosms containing cells added in a sterile soil carrier without preincubation or preincubated for 7 days at  $1 \times 10^9$  cfu  $g^{-1}$  (Fig. 2A) or added in a sterile soil carrier without preincubation at  $1 \times 10^8$  cfu  $g^{-1}$  (Fig. 2B) had significantly higher activities than control treatments at days 0 and 2 after inoculation. There were no differences in activity between treatments at day 4, and after day 4 the activity of all treatments was below the background level of  $\log 1.6$  rlu  $g^{-1}$  soil. Close association with nutrients available in the sterile soil carrier could enhance activity of *P. fluorescens* for a short time following inoculation into soil microcosms, but the addition of nutrients by pre-mixing soil microcosms with 1 or 10% sterile soil did not enhance activity of *P. fluorescens*. These results indicate that cell activity decreased rapidly in non-sterile soil following inoculation with either a colonised sterile soil carrier or a liquid cell suspension.

## 4. Discussion

We have tested a simple strategy in which bacterial survival is enhanced by incubating cells in sterile, autoclaved soil prior to introduction into non-sterile agricultural soil. *P. fluorescens* was selected for use as it allows comparison with a number of other studies monitoring survival under various environmental conditions in soil (for example, Crowley et al., 1996; Meikle et al., 1995; Van Dyke and Prosser, 1998; van Overbeek et al., 1995). In addition, fluorescent pseudomonads have been proposed as bacterial soil inoculants for applications such as plant growth promotion

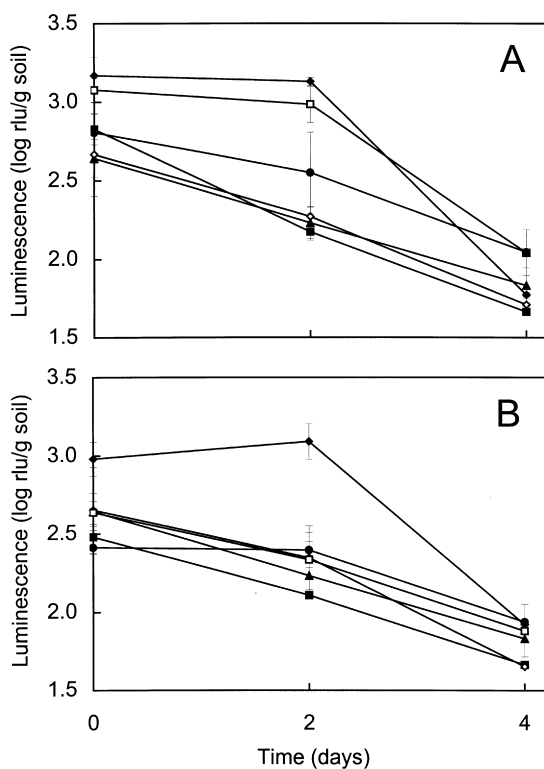


Fig. 2. Luminescence activity of *P. fluorescens* MON787 in soil following inoculation with 1% (A) or 10% (B) colonised sterile soil carrier. Treatments and symbols are as described in Fig. 1.

(Glick, 1995; O'Sullivan and O'Gara, 1992) and pollutant degradation (Brazil et al., 1995; Caldini et al., 1995).

A decline in the concentration of cells introduced into soil can reduce the effectiveness of bacterial inoculants, and fluorescent pseudomonads are known to survive poorly in non-planted agricultural soils. A survey by van Elsas and van Overbeek (1993) reported decline rates of fluorescent pseudomonads in various soil types from 0.2 to 1.1 log units per 10 day period, although larger rates have been determined (Araujo et al., 1994; Blackburn et al., 1994). When free bacterial cells are added to soil, survival can depend on their location within the soil matrix. The addition of bacterial inocula to carrier materials may enhance cell survival by physically separating bacteria from environmental influences, through attachment to a surface or pore space favourable to survival and growth and/or by providing nutrients (van Veen et al., 1997).

Storage and preincubation of *P. fluorescens* MON787 in a sterile soil carrier before addition to non-sterile soil resulted in higher survival than addition of free cells to soil in a liquid suspension (Fig. 1A and B). Preincubation in sterile soil for 1 or 2 weeks before addition to non-sterile soil resulted in a decrease of only 2 log units after 1 month, and cells preincubated for 2 weeks remained detectable at  $10^3$  cfu  $g^{-1}$  soil after 2 months. *P. fluorescens* added to soil in a liquid suspension was not detectable after 1 month. Experiments using peat carriers for rhizobia inoculants have similarly reported higher survival and nodulation using sterile rather than non-sterile material (Strijdom and van Rensburg, 1981). Increased maturation times of legume inoculants in sterile and non-sterile peat have been shown to enhance survival of bacteria on plant seeds (Burton, 1976; Thompson, 1980).

Sterilisation of soil by autoclaving is known to release nutrients that can be utilised for bacterial growth. However, nutrient addition alone cannot explain the enhanced survival of *P. fluorescens* in soil, since control microcosms containing soil premixed with sterile soil did not enhance cell survival (Fig. 1A and B). In addition, there was no significant difference in luminescence activity, following addition to soil microcosms, between control treatments and cells that were preincubated in sterile soil for 2 weeks. This indicates that the nutrient supply in the sterile soil carrier was depleted and cells were nutrient stressed.

Growth of *P. fluorescens* cells in the sterile soil carrier may have allowed bacteria to adapt and develop resistance to stress conditions encountered in soil. In preparing high cell numbers for microbial inocula, growth in liquid culture medium using readily degraded substrates is used. This results in a culture whose metabolism is not adapted to the

different carbon sources and starvation conditions that exist in most soils. In soil, bacterial adaptation to stress conditions was demonstrated by van Overbeek et al. (1995), who found that exponential phase *P. fluorescens* R2f cells inoculated into soil developed increased resistance over time to normally lethal factors, such as osmotic tension and oxidative stress. Although carbon starvation of *P. fluorescens* R2f in minimal medium before addition to soil did not enhance survival (van Overbeek et al., 1995), the altered physiological state of *P. fluorescens* MON787 preincubated in sterile soil could have allowed cells to survive for longer periods of time. Studies performed by Megharaj et al. (1997) have similarly found that *Sphingomonas* sp. preadapted to soil conditions survived better and degraded pollutants faster than non-adapted samples after inoculation to soil microcosms.

Establishment of bacteria in soil particles may also have resulted in higher survival of *P. fluorescens*. Increased attachment to the soil matrix due to reduced competition in sterile soil, and growth following preincubation of *P. fluorescens* for 7 and 14 days, could result in microcolony formation. In addition, increased survival of bacteria preincubated in a sterile soil carrier could be a result of protection from protozoan predation. Wright et al. (1995) found that selective addition of *P. fluorescens* to small-sized pores in soil protected cells from predation by added protozoa compared to cells added to intermediate-sized pores. These factors, together with nutrient adaptation, may all have contributed to increased survival of *P. fluorescens* in soil.

The use of polymeric carrier materials to encapsulate bacteria before soil inoculation have proven successful in enhancing survival of bacteria (Briglia et al., 1990; Cassidy et al., 1997; Leung et al., 1995; Trevors et al., 1993; Weir et al., 1996). The reasons for increased survival using polymeric materials is not always explored, although the addition of nutrients, surface attachment, high cell density and physical separation from soil and environmental stresses may all be contributory. Results using polymeric materials are promising, but direct comparisons with simple methods, such as sterile soil carriers, are required in terms of bacterial survival, target response, simplicity of use and production cost.

The method of using a sterile soil carrier for the introduction of bacteria into soil is simple, low cost and effective for increasing cell survival. Preincubation of bacteria in sterile soil before addition to non-sterile soil was shown to enhance survival of *P. fluorescens* significantly compared to addition of a liquid cell suspension. Factors such as the development of stress resistance, increased attachment to soil particles, and protection from protozoan predation, may have contributed to enhanced survival of *P. fluorescens* in soil.

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