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Density independent population dynamics by *Trichoderma virens* in soil and defined substrates

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Abstract
Classical models of population dynamics predict that with increasing initial population densities the *per capita* growth will diminish. Observations over a broad range of initial densities with a wild-type and a genetically engineered strain of the filamentous fungus, *Trichoderma virens* (Arx), in soil and autoclaved soil differed from these predictions. The *per capita* growth response of *T. virens in vitro* was found to be density dependent on potato dextrose agar, but density independent on water agar. Further experiments with a defined, carbon-free medium (Vogel's medium) and, with the same medium containing sucrose, indicated that density dependent *per capita* growth occurred in the nutrient-rich medium but not the oligotrophic medium. This hypothesis was tested and supported experimentally through observation of density dependent *per capita* growth after adding nutrients to autoclaved soil. Development of better models of population dynamics will be important to predict successfully the likelihood and extent of establishment after field release of microorganims.

Keywords: Trichoderma virens, *population regulation, biocontrol fungi*

Introduction

Trichoderma virens (Arx) is a common soil saprophyte and mycoparasite with a long and extensive history as a biocontrol agent (Weindling 1932; Harman 1990). Transgenic strains of *T. virens* with enhanced levels of biocontrol activity against soil-borne seedling diseases are in development (Baek et al. 1999; Pozo et al. 2004). Similar efforts are proceeding with other species of *Trichoderma* for other pathosystems (Chet et al. 1993; Migheli et al. 1994; Bowen et al. 1996). It is important to develop models that are useful in predicting the spatial and temporal population dynamics of any transgenic strains that might be deployed in the agroecosystem.

A useful starting point for such modeling efforts is the Verhulst-Pearl (Pianka 1994; Ricklefs 1993) logistic growth equation. While the Verhulst-Pearl equation makes assumptions that are unlikely or impossible (Pianka 1994), it serves as a good model for understanding some of the determinants of population dynamics. Density dependence is a ubiquitous element of population models (Pianka 1994; Ricklefs

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1993) and is a mechanism by which population growth rates diminish as the population size approaches the carrying capacity.

The most common means of determining carrying capacity is by analysis of time series data (Berryman & Turchin 2001; Turchin 2001). A time series analysis assumes that when a population is above the carrying capacity, it will tend to decrease at the next time point and if it is below the carrying capacity, it will tend to increase at the next time point. McCallum (2000) described the various statistical means to analyze time series data to establish density dependence and noted that all of them are *ad hoc* and subject to various criticisms. He advocated, when possible, a manipulative experimental approach. One such approach is to inoculate the system with a range of densities of primary inoculum and to measure the *per capita* growth, i.e., the increase in the population size, normalized by dividing by the starting population size. If the population is being regulated by the carrying capacity, then the *per capita* growth will decline at higher population densities. This method was employed to measure the carrying capacity of soil typical to where engineered strains of *T. virens* might be deployed (Weaver et al. 2005). In that study, high fungal populations introduced into soil slowly declined over a long time period. While this system was useful for evaluating the persistence of the populations, a different system was needed to consider population growth. Alginate prills, a formulation used for deployment of soil-borne fungi, satisfied this requirement. *T. virens* grows robustly from this inoculum; the prills have a long shelf life; it is easy to manipulate starting densities and they permit spatially explicit experiments (Knudsen & Bin 1990).

The experiments described here were developed in response to a series of unexpected observations from studies on the long term survival, responsiveness and competitiveness of a genetically engineered strain of *T. virens* GvT6 relative to its near-isogenic, parental strain, 29-8 (Weaver & Kenerley 1999; Weaver et al. 2005). Experiments intended to evaluate the competitiveness of the two strains were included in this work. These experiments involved incorporating mycelia and chlamydospores of each strain into agriculture soil individually and as a mixture so that the two strains would compete over time. In the analysis of these experiments, there was no evidence that the presence of one strain had a negative impact on the other, and no evidence that the two strains even interacted. These results challenged the assumptions of density dependent population regulation.

In preliminary experiments to demonstrate density dependent growth, we were unable to observe a decreased *per capita* growth rate with increased prill density, even over a wide range of initial densities (Weaver & Kenerley, unpublished). While there is precedent for this observation, the alternative mechanisms of population regulation, such as predation or environmental stochasticity, did not seem appropriate. The following experiments and observations describe our efforts to understand the regulation of *T. virens* population densities in soil.

Methods

Strains and cultural conditions

Two wild type *T. virens* strains, 29-8 and 4-2D were isolated from agricultural sandy loam soil near College Station, TX (Park et al. 1992a) and maintained on potato dextrose agar (PDA; Difco Laboratories). The genetically engineered strain, GvT6, was derived from the wild type strain 29-8 by transformation and heterologous

integration of the plasmid pCL1, which included the hygromycin resistance gene *hygB* and a gene encoding organophosphate hydrolyase (OPH), *opd*. GvT6 was selected based on high expression levels of the OPH and stable hygromycin resistance (Dave et al. 1994). Conidia were collected by scraping 1-week-old cultures grown on PDA with water and filtering the suspension through miracloth (Calbiochem). For chlamydospore production, conidia were added to molasses medium shaken at room temperature (Papavizas et al. 1984) and biomass was harvested after 14 days by vacuum filtration over miracloth. A medium selective for *Trichoderma*, GVSM, was developed based on Park et al. (1992b).

Production of alginate prills

Conidia or chlamydospores were incorporated in a solution containing 1% sodium alginate (medium viscosity, Sigma A2033), 20% polyethylene glycol (PEG 8000, Fisher BP233) and 2% ground wheat bran (all on w/w basis) (Daigle & Cotty 1997; Boyette & Walker 1985). The solution was allowed to drip through large orifice pipette tips into a 0.25M solution of calcium chloride. The resulting prills were spread over plastic mesh and dried overnight in a laminar flow hood. The alginate solution contained 1×10^8 conidia g^{-1} or 6 g (wet weight) of chlamydospores g^{-1} alginate solution.

Density dependence in soil

Soil was obtained from a cotton field near College Station, TX which has been characterized (Baek & Kenerley 1998). Soil was passed through a 1.8 mM sieve and adjusted to -0.05 MPa, based on a soil moisture release curve. In experiments with sterile soil, the soil was autoclaved on two successive days with 1% potato dextrose broth (PDB) (w:w) added before the second autoclaving. Microcosms were established in circular, 348 mL polypropylene containers (Rubermaid 552A1) containing 100 g of soil. In experiments with 26, 48, 75 or 129 alginate prills, the prills were added in an evenly spaced grid corresponding to 1.5, 1, 0.8 or 0.6 cm inter-prill distances, respectively. In experiments with up to 20, 40, 60, 80, 120 or 200 prills the soil and prills were mixed. All experiments were incubated at 27°C. After incubation for the indicated time periods, the microcosms were emptied into beakers and mixed. Samples (5 g) were collected and mixed with 45 mL of water in a Waring blender. Serial dilutions were made and samples spread on GVSM. After 2 days incubation, population sizes were determined by counting colony forming units (conidia and mycelia) on GVSM plates.

Density dependence in vitro

Prills containing conidia were placed on solid medium in 60 mm Petri dishes containing 10 mL of Vogel's minimal salts (VMS) medium (Vogel 1956, 1964) supplemented with 2 mM arginine or VMS supplemented with 2 mM arginine and up to 1.5% sucrose (VMS+S) or PDA. Plates were incubated at 28°C. After 4 days incubation, each plate was blended with 90 mL of water. Serial dilutions were made and samples spread on GVSM. After 2 days incubation, population sizes were determined by counting colonies on GVSM plates.

Analysis

To obtain a *per capita* growth response, the number of CFUs found by plating on selective media was divided by the number of prills added as inoculum. This *per capita* response was regressed against the starting density and the hypothesis that this slope was equal to zero was tested using Statview 5.0.1 (SAS Institute).

Results

Growth in soil

The growth from the alginate prills containing chlamydospores was measured after 7 days incubation at 28°C in non-sterile soil (Figure 1A). With increased inoculum, there was increased growth, in a linear relationship (29-8 $R^2 = 0.95$; GvT6 $R^2 = 0.79$). The *per capita* growth increased with higher inoculum levels (Figure 1B). There are several plausible reasons for why the growth might deviate from the pattern predicted by the density dependent models. To evaluate some of these explanations, the experiment was repeated with sterile soil, conidia instead of chlamydospores in the prills, a second wild-type strain of *T. virens* (4-2D), longer incubation, and an even

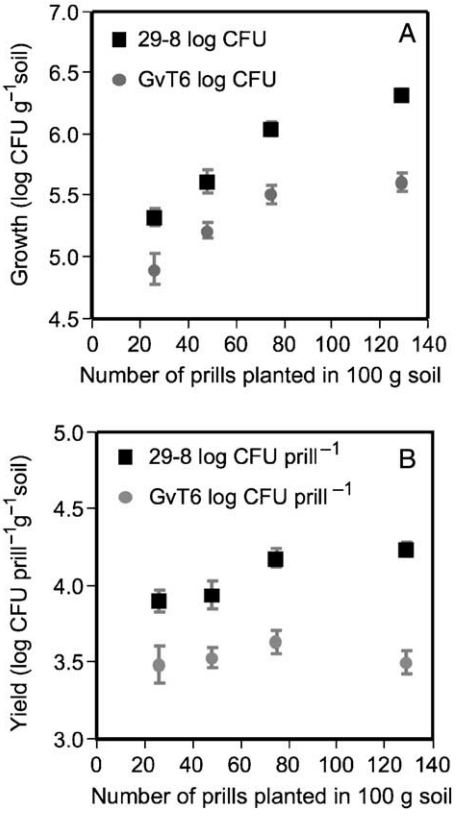

Figure 1. Growth and yield response after 7 days growth of *T. virens* prills planted in non-sterile soil. (A) Growth of *T. virens* strains 29-8 and GvT6 as a function of the density of prills planted in soil. (B) Yield, or *per capita* growth, of *T. virens* strains 29-8 and GvT6 as a function of the density of prills planted in soil. Error bars indicate 2 × standard error of the mean.

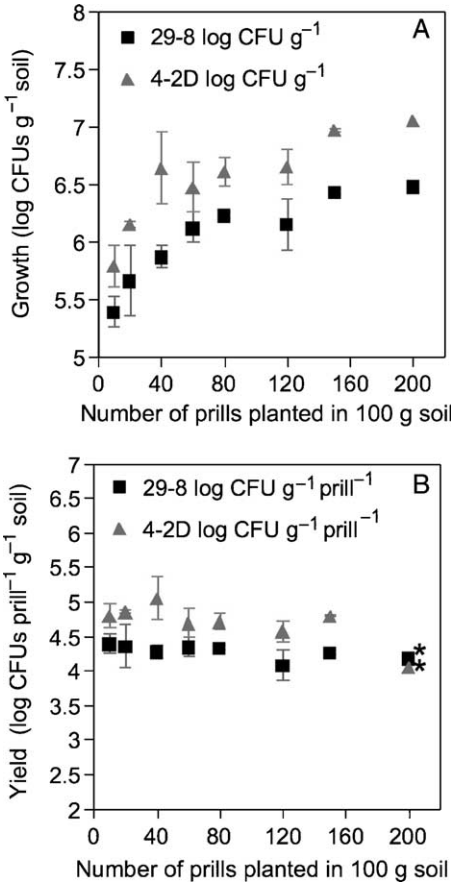

Figure 2. Growth and yield response after 14 days growth of *T. virens* prills planted at high density in sterile soil. (A) Growth of *T. virens* strains 29-8 and 4-2D as a function of the density of prills planted in soil. (B) Yield, or *per capita* growth, of *T. virens* strains 29-8 and 4-2D as a function of the density of prills planted in soil. Error bars indicate one standard error of the mean. * indicates a slope not significantly different than zero.

higher prill density. To facilitate this higher density, the prills were not planted on the uniform grid, but mixed into the soil. After 14 days incubation growth was measured. As in the previous experiment, the growth was higher with increased inoculum levels (Figure 2A). The *per capita* growth (Figure 2B), however, did not decline over the broad range of densities examined for either strain (slope not significantly different than zero).

Growth in vitro

The growth of *T. virens* strain 29-8 *in vitro*, is presented in Figure 3. The growth on PDA was more than twice as much as VM+S (Figure 3A and B, respectively), but the pattern of growth was the same on both media. While the population density generally increased with higher initial population densities, the yield, on a per prill basis declined with higher initial population densities. This is in contrast with the patterns of growth on the two oligotrophic media, VM and water agar (Figure 3C and D,

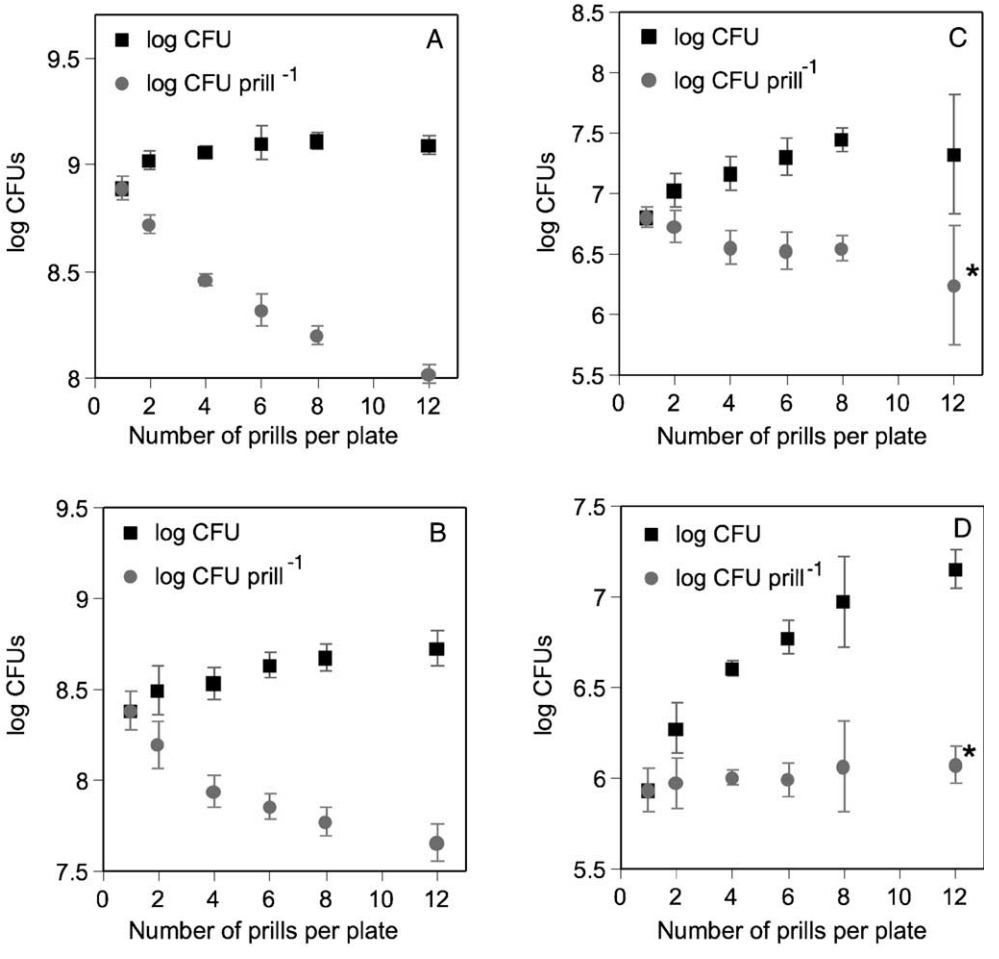

Figure 3. *In vitro* growth of *T. virens* prills. Growth and *per capita* yield of *T. virens* strains 29-8 on (A) PDA (B) VM+S (C) VM and (D) water agar. Error bars indicate one standard error of the mean. * indicates a slope not significantly different than zero.

respectively). On these nutrient deficient substrates, the growth steadily increased with higher initial population while per prill yield was constant.

These results were consistent with the hypothesis that per capita density dependent growth is linked to the nutrient level of the medium. To explore this relationship further, prills of GvT6 were incubated on VM without additional sucrose, with 0.5, 1.0 and 1.5% sucrose. There was a strong response to the sucrose, with the total growth at the 1.5% concentration ~30 times higher than the level of growth without sucrose (Figure 4). There was also a density dependent population response on media that included sucrose, with a negative relationship between per prill yield and prill density.

Growth in sterile, amended soil

The observations of growth *in vitro* are consistent with the hypothesis that the failure to observe density dependent *per capita* fitness can be explained by the oligotropic

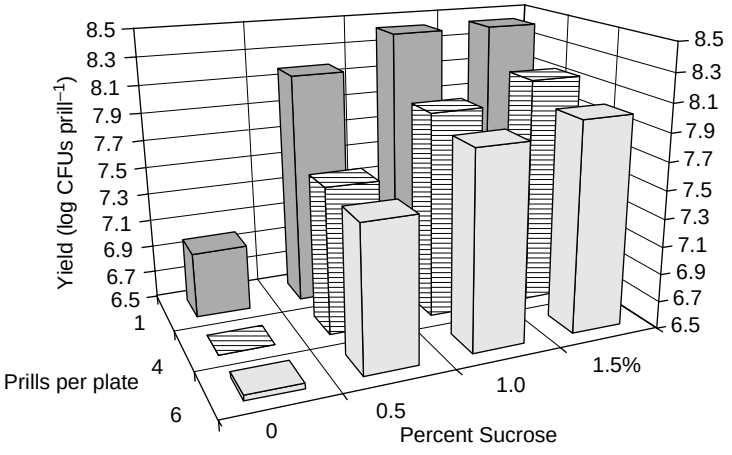

Figure 4. Yield of prills on VM with varying levels of sucrose.

condition of the soil. The growth in soil amended with potato dextrose broth (PDB) (Figure 5) was consistent with the patterns predicted by the classical ecology models, and similar to that observed *in vitro* on carbon-rich media (Figure 5A and B).

Discussion

There are many possible hypotheses that could explain the disagreement between the predictions based on density dependent population regulation and our observations of the growth by *T. virens* from alginate prills in non-sterile soil. Other common mechanisms for regulation of populations are regulation by predator-prey dynamics or environmental fluctuations, such as Andrewartha's classical experiments with thrip populations that were explained by changes in the weather (Andrewartha & Birch 1954). Neither predator-prey interactions nor environmental variability are capable of explaining the observations of density independent *per capita* growth by *T. virens* in homogenized, sterilized soil in incubated containers. Additional experiments are necessary to evaluate other explanations for the divergence between the theoretical growth response and the observations of apparent density independent *per capita* growth.

It was conceivable that the initial densities examined in the first experiment were so low that the populations never approached the carrying capacity. For example, there could have been a biological activity from the soil that influenced the growth response of *T. virens*. It could be suggested that there was something about the alginate prill formulation or this particular wild-type strain that lead to the unexpected growth response. To evaluate these hypotheses, the alginate prills were reformulated to include higher initial densities and a second wild-type strain, and the soil was sterilized immediately prior to introducing the inoculum. Initial densities were increased to span greater than a log order and grossly exceeded any natural population levels. Still, the observed *per capita* fitness did not respond in a density dependent manner.

To understand better the basis for the unexpected population dynamics and to reduce the environmental complexity, the experiment was repeated *in vitro*. The results on PDA, a rich, artificial medium were consistent with the density dependent models that invoke the mechanism of exploitative competition for some limiting

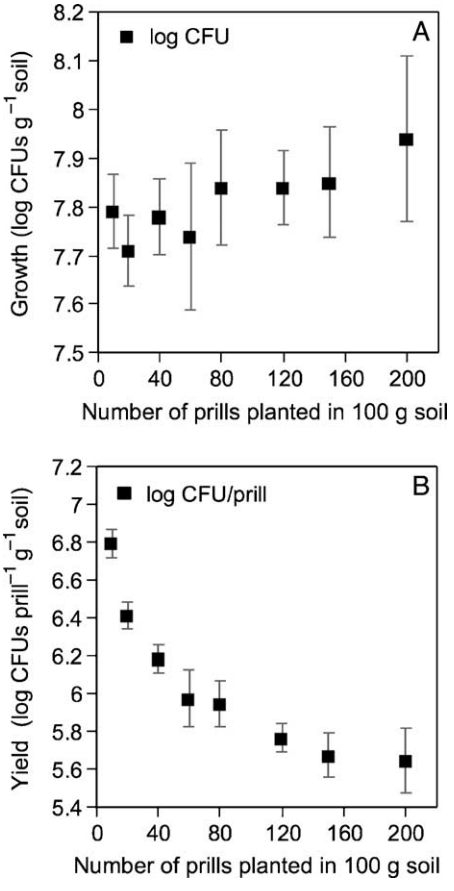

Figure 5. Growth (A) and *per capita* fitness (B) of *T. virens* strain 29-8 from prills containing condia in soil supplemented with PDB. Error bars indicate one standard error of the mean.

resource. The fact that the same fungus, from the same inoculum source, displayed density dependent *per capita* growth *in vitro*, but not in soil or in sterilized soil indicates that the observations in soil are not an artifact of the organism, or the inoculum source or the method to measure growth.

There are many differences between growth in soil and growth on PDA. We hypothesized that the differences in *per capita* growth response could be explained by the oligotrophic nature of agriculture soil. Observations of the *per capita* growth response *in vitro* on water agar were consistent with this hypothesis. The observation that the *per capita* growth response on VM was density independent while it was density dependent on VM + S, supports the view the growth response of *T. virens* from prills can be manipulated simply by changing the quantity of available carbohydrates. The same relationship was found with prills of strain GvT6 on VM with varying levels of sucrose; on carbon-rich media final populations were greater and the per prill growth was density dependent.

The *in vitro* observations are consistent with the hypothesis that the density independent *per capita* growth in soil is a result of the oligotropic nature of the soil. To test this hypothesis more directly, the nutrient base of PDA was added to sterile soil

with known numbers of alginate prills of strain 29-8. The observed *per capita* density dependence when nutrients were added to the soil adds support to the view that the previously observed density independence is the result of the oligotrophic nature of the soil.

Many other systems that appear to violate the model of density dependent *per capita* fitness can be treated as "special cases." For example, Andrewartha's thrip populations' oscillations were explained by changes in the weather (Andrewartha & Birch 1954). Many other systems are regulated by relatively complex predator-prey interactions. It has been suggested that the failure to detect density dependence in some systems is an artifact of sampling size and highly heterogeneous environments (Ray & Hastings 1986). Our observation of density independent growth cannot be readily dismissed as a "special case" and appears to avoid some of the artifacts of other systems. In an enclosed, temperature controlled microcosm of sterilized soil, with an introduced population of a known size we have avoided many of the previous "exceptions." Since our microcosms were mixed and the samples were so large relative to the environment perceived by a microorganism, it seems unlikely that our observations are simply an artifact of sampling methodology. The fact that *T. virens* exhibited growth that was consistent with density dependent *per capita* fitness in some environments and not in others that differed only in the nutrient availability suggests the problem is not with the methodology employed to measure the population. Our failure to detect density dependence is clearly not an artifact of using 'CFUs' as a proxy for the population size. If it were simply an artifact, then it would be seen *in vitro* on all media.

It could be suggested that, because density dependence exerts only a small influence until the population approaches carrying capacity, that our failure to observe density dependence means that our data set only included populations that were well below the carrying capacity. We reject this view because our observed population densities (>1 million CFUs g^{-1} soil) are considerably higher than generally reported in bulk soils, and it is difficult to imagine the value of adding more than 2 prills g^{-1} of soil. In fact, in the *in vitro* experiments, density dependence was evident with starting densities below one prill g^{-1} of substrate.

We are unwilling to reject the model of density dependent population regulation, particularly because our observations in some environments fit it so well. However, in other environments, such as in bulk soil, there are disagreements between the predictions made by the model and our observations. It seems reasonable that the population might be regulated by nutrient availability. In the case of sterile soil, water agar and VM agar, the *per capita* fitness was constant over a large range of inoculum densities. We propose that each additional input of inoculum, a prill in this case, also provided a small additional nutrient input. In the case of PDA, VM+S and soil amended with PDB, this small input was trivial relative to the nutrients already in the environment. Thus, in the oligotrophic environments the additional inoculum might alter the carrying capacity.

Our conclusions are not a rejection of density dependence. It is plausible that the carrying capacity in bulk soil is very low and the introduction of inoculum has significantly changed that carrying capacity. Thus, the population might generally be regulated by density dependent mechanisms as described by Verhulst-Pearl. In our experimental system, and likely in the case of field deployment, the robust growth of

the inoculum might, at least in the short term, overwhelm the negative effect of density dependent population regulation.

One implication of this form of population regulation is a diminished role for competition in bulk soil. Competition may still be important in the rhizosphere or on plant debris as it is incorporated into the soil or on other nutrient-rich patches. If density dependent *per capita* fitness cannot explain the population dynamics of *T. virens* in soil, then there is little potential for intraspecific competition to exert an influence. We are then left without a biologically meaningful model for predicting population dynamics. It has been suggested that genetically engineered organisms are inherently less competitive (Davies 1988). If this is true, it might provide a measure of safety when assessing likelihood of escape or persistence of genetically engineered organisms. However, if in some environments, such as agricultural soil, intraspecific population regulation is unimportant, then this margin of safety is negated. Furthermore, the very assumption that transformants are less fit or less competitive is debatable (Lenski 1993). We conclude that the likelihood of establishment of populations of genetically engineered organisms in the agroecosystem has been underestimated.

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