Experiences in the Molecular Ecology of the Rhizosphere with Microbial Releases.

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Abstract

The ability of certain fluorescent pseudomonad strains to suppress plant fungal diseases has been linked to production of secondary metabolites such as 2,4-diacetylphloroglucinol (Phl). Genetic modification of biocontrol strains for increased production of antifungal metabolites has allowed the development of strains with improved biocontrol capabilities. Within the EU BIOTECH IMPACT project we have developed a multifaceted approach for the evaluation of wild type biocontrol strain such as *P. fluorescens* strains F113 on suppression of "damping off" of sugarbeet and the impact on non-target resident microbial populations under field conditions. The rationale of these studies is to utilize wild type biocontrol strains to develop a baseline against which GM strains may be evaluated. The resident microbial populations of fluorescent pseudomonads and *Rhizobium meliloti* were chosen as indicators to evaluate the impact of F113 on non-target microbial species. Results of phenotypic and genotypic analysis revealed no significant alterations in the composition of fluorescent pseudomonads as a result of inoculation by F113. In addition, no adverse effects on the indigenous population of *R. meliloti* were detected with regard to nodulation and plant performance of uninoculated red clover planted in a crop rotation scheme. We have also developed a containment system for rhizobial inoculants based on a stable mutation of the essential gene *thyA* encoding thymidylate synthase in *R. meliloti*. In addition, this system may also be used to evaluate the risk of gene transfer from inoculants to indigenous microbial populations.

Introduction

The use of microbial inoculants as biological control agents of plant diseases is an increasingly important area in agricultural research. The interest in the use of microbial inoculants addresses concerns associated with the use of synthetic chemical pesticides in current agricultural practices. Due to increasing concern regarding the safety of synthetic chemical pesticides, restrictions have been placed on the use of a number of fungicides for the control of fungal root diseases and tighter regulations including total bans on certain fungicides may be expected in the future. Strains of *Pseudomonas fluorescens* have been identified which exhibit antifungal activities against a number of soil-borne fungal pathogens under laboratory conditions. These strains have been the

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focus of research into protection of plants from soil-borne fungal pathogens (Cook, 1993; Cook *et al.*, 1995; Fenton *et al.*, 1992; Maurhofer *et al.*, 1994; Thomashow and Weller, 1988). Examination of the mechanisms whereby these strains exert their antifungal properties has revealed a close association between biocontrol and the production of antifungal secondary metabolites including phenazines and phloroglucinols (Cook, 1993; Dowling and O'Gara, 1994; Keel *et al.*, 1992; O'Sullivan and O'Gara, 1992; Russo *et al.*, 1996; Shanahan *et al.*, 1992). A number of additional metabolites have also been implicated in biocontrol including iron-chelating siderophores, HCN, and salicylic acid (Dowling and O'Gara, 1994; Keel *et al.*, 1992).

P. fluorescens strain F113 which was isolated from the rhizosphere of sugarbeets shows a biological control activity against *Pythium ultimum,* the causative agent of the "damping off". The biocontrol capability of F113 has been linked to the production of the metabolite 2,4-diacetylphloroglucinol (Phl) (Carroll *et al.*, 1995; Dowling and O'Gara, 1994; Fenton *et al.*, 1992). A mutant derivative, F113G22, is deficient in Phl production and has lost the ability to protect sugarbeets from damping-off in soil microcosms. Introduction of a plasmid containing the Phl biosynthetic genes into F113G22 restores Phl production and biocontrol ability. A major focus of research in biological control is the generation of genetically modified inoculant strains with improved biocontrol abilities. The antifungal nature of secondary metabolites such as phenazines and phloroglucinols has made them prime targets for genetic manipulation. Genetically modified derivatives of *P. fluorescens* biocontrol strains with enhanced production of Phl and pyoluteorin (CHA0) and Phl (F113) have been obtained which exhibit increased antifungal activity *in vitro.* Inoculation of cucumber with the modified CHA0 strain resulted in a significant increase in protection against *P. ultimum* (Schnider *et al.*, 1995).

The use of biocontrol strains in the effective control of fungal plant pathogens involves the introduction of large numbers of bacteria into the environment. The ecological impact asociated with the deliberate release of large numbers of biocontrol strains, especially those genetically modified to overproduce antifungal metabolites such as Phl needs to be evaluated. In our field studies, we have utilized F113Rif, a spontaneous rifampicin-resistant isogenic mutant of F113 in which Phl production and biocontrol ability are not affected. The rationale of these studies is to evaluate the impact of the wild type biocontrol strain on selected resident microbial communities in order to develop a baseline against which genetically modified derivatives may be compared. Ecological effects were determined at a number of levels, including the soil microbial community as a whole, pathogenic microoorganisms (i.e. *P.ultimum*), and selected beneficial microbial strains (*Rhizobium* and *P. fluorescens*).

Impact of *Pseudomonas* inoculants on the total soil microbial community

The resident microbial community is an essential soil component which contributes to the fertility of soil, thereby influencing crop yield and plant health. Evaluation of the impact of biocontrol inoculants such as F113 on key microflora may be achieved

through measurements carried out on the field crop which serves as a "biosensor". Soil microorganisms, especially bacteria play a significant role in biogeochemical cycles (i.e. carbon, nitrogen, phosphorus, sulfur, etc.). Perturbations associated with the use of biocontrol strains on the resident microflora which is involved in these processes may be reflected through reductions in soil fertility and hence plant yield parameters. Field trials designed to evaluate the performance of microbial inoculants on sugarbeet have been carried out in a joint collaboration between UCC and Irish Sugar Plc. These trials have evaluated the wild type *Pseudomonas fluorescens* biocontrol strain F113 which was originally isolated from the rhizosphere of sugarbeets. Evaluation of the biocontrol ability of this strain in microcosm studies demonstrated that inoculation of sugarbeet seeds with this strain affords significant protection against "damping-off" induced by *Pythium ultimum.* Appropriate controls were included in these studies which consisted of the use of a proprietary seed pelleting mix formulated by Irish Sugar Plc in which one control contains a commercial fungicide (i.e. Thiram), unlike the other control.

Field trials were set up in a Latin square arrangement to minimize field heterogeneity and the level of infestation by the pathogen was determined for each site. Following sowing, the sites were monitored on a regular basis for sugarbeet emergence. The effectiveness of the inoculant strain was determined by comparison to emergence obtained with the fungicide-free control. We did not observe any significant difference in emergence between seeds inoculated with F113 and the controls. The absence of a significant difference in emergence between the two controls indicated a lack of disease pressure in the fields (Fig. 1). This apparent lack of disease pressure in the presence of relatively high levels of the pathogen (i.e. 1000 propagules/g soil) prevented a critical evaluation of the biocontrol capabilities of the inoculant strain. While the inability to accurately predict disease prior to sowing is a serious impediment to field evaluations of inoculant strains with regard to biocontrol of "damping off" of sugarbeet, these conditions provide ideal conditions with which to evaluate the inoculant for detrimental effects on sugarbeet. Evaluation of sugarbeet at harvest for plant performance parameters such as root yield, sugar content, and recoverable sugar indicated that inoculation with F113 did not result in significant detrimental effects on sugarbeet. However, measurements of plant yield may not be sensitive enough to detect small or transient effects on soil fertility. Analysis of soil for changes in biochemical characteristics such soil enzymes and nutrient content may reveal effects of inoculants which do not translate in terms of plant yield. Measurements of soil nutrient levels were carried out at a site inoculated in 1994 with F113 and sown with red clover one year later. No significant differences were observed for any parameter measured between the three treatments. On this basis, it appears that inoculation of sugarbeet with F113 did not result in any significant alteration in the fertility of the soil.

Impact of inoculants within normal agricultural practice

In 1994, a field trial designed to evaluate a number of biocontrol strains including

F113 was carried out at a site located near Bandon, Ireland. The overall purpose of this study was to evaluate the ecological impact of F113 under normal agricultural practices. One year after the release of F113 as seed inoculation on sugarbeet, uninoculated red clover was sown at the same site in a crop rotation cycle. The impact of F113 inoculation on the resident populations of fluorescent pseudomonads and *Rhizobium leguminosarum* bv. *trifolii* was evaluated.

Impact of inoculants on indigenous populations of fluorescent pseudomonads

In addition to the evaluation of the biocontrol potential of strain F113 based on emergence and plant yield, indigenous fluorescent pseudomonads were isolated from the rhizosphere and rhizoplane of inoculated and uninoculated plants. No significant differences were observed in colonization of the rhizosphere of sugarbeets by total aerobic bacteria between F113-inoculated plants and the uninoculated control. The total aerobic bacterial counts included a variety of aerobic bacteria including fluorescent pseudomonads. At one month after release, F113 accounted for 10% of the total aerobic bacteria isolated from the rhizosphere and the number declined thereafter, F113 accounted for 0.1% of the total aerobic bacteria at three months (Fig. 2). Within six months the amount of inoculant had declined to below detection limits. This decrease in the relative numbers of F113 is a desirable characteristic for biocontrol inoculants as plants are most susceptible to "damping off" during the early stages of development.

At 19 days after sowing, a total of 499 indigenous fluorescent pseudomonads were isolated from the rhizosphere and rhizoplane of uninoculated and F113-inoculated plants, as well as from bulk soil from Bandon. These isolates were subjected to phenotypic analysis using 43 assimilation tests and resistance to 8 antibiotics. In addition, genotypic analysis utilising ARDRA (Amplified Ribosomal DNA Restriction Analysis) and RAPD (Random Amplified Polymorphic DNA) was carried out on these isolates. These analyses were designed to detect any subtle alterations in the diversity of the indigenous population of fluorescent pseudomonads which would not be reflected in plant yield or colonization data. A total of 434 phenotypic profiles were detected, along with 291 RAPD profiles. No significant difference in the resistance profiles of the isolates to Phl could be detected between the two treatments. The most frequent phenotypic profile was found in only 2% of the isolates and the most frequent RAPD profile was found in only 4% of the isolates. These results indicate a surprisingly high degree of diversity in the indigenous population of fluorescent pseudomonads not detected previously in similar studies. Studies designed to determine the significance of these results and to relate the degree of phenotypic diversity with the genotypic data are continuing.

Impact of inoculants on indigenous populations of *Rhizobium leguminosarum* bv. *trifolii*

Our studies also employed indigenous rhizobial species as potentially useful indicator soil organisms to detect any possible negative effects associated with the use of biocontrol inoculants. These strains are important in agriculture due to their nitrogen-fixing symbiotic associations with legume crops. Any negative effects on the indigenous populations of Rhizobia should be detected through plant parameters such as nodulation of the root along with plant yield or nitrogen content. In 1995 we utilized uninoculated red clover as a "biosensor" crop to detect perturbations in the indigenous population of *R. leguminosarum* bv. *trifolii.* at Bandon. Clover was sampled on two occasions and several plant parameters were examined, including nodulation and a quantitative and qualitative analysis of clover shoots. There were no significant differences in any of the yield parameters examined between the controls and F113 with regard to yield (Fig. 3) and nitrogen content. No significant effects were observed in terms of nodulation of clover as a result of the F113 treatment. These results indicate that the use of F113 as an inoculant does not appear to adversely affect the native *Rhizobium* population interms of plant yield, health and nodulation. In addition, isolates of *R. leguminosarum* bv. *trifolii* have been obtained from these nodules and will be subjected to phenotypic and genotypic analysis as in the case of the resident fluorescent pseudomonad population. This will allow us to detect subtle alterations in the diversity of the *Rhizobium* population as a consequence of introduction of the biocontrol strain F113.

Development of a containment system in *R. meliloti*

Genetic manipulation of *Rhizobium* strains is a promising method for the development of strains with improved symbiotic performance(Bosworth *et al.*, 1994 ; Chen *et al.,* 1991; Triplett, 1990). Most genetic modifications will likely involve the use of plasmids which will be introduced into the rhizobial strains. Stability of introduced plasmids is a serious concern as in the absence of selection, the introduced plasmid tends to be lost over time. A possible solution is to combine a strain with a mutation in an essential gene with a plasmid carrying a copy of that gene. This would improve stability as cells which lost the plasmid would not survive. The development of a biological containment system based on the conditional expression of the essential host gene *thyA* encoding thymidylate synthase offers a potential for the biological containment of *Rhizobium* inoculants. Thy mutants of *R. meliloti* are characterized by their inability to nodulate their host plant alfalfa. In addition, these mutants have limited ecological fitness as they are unable to survive in the rhizosphere of non-host plants such as sugarbeet and in the thymidine-deficient environment of soil. Results previously reported by our group have demonstrated the effectiveness of this system under field conditions (O'Flaherty *et al.*, 1995). These studies utilized spontaneous thy mutations which are limited by a relatively high reversion frequency. A broad host range

plasmid containing a copy of the *thyA* gene from *Lactococcus lactis* completed the autoselective system. This system was evaluated in a field trial at Fota, Ireland. This site is characterized by the absence of indigenous populations of *Rhizobium meliloti*. Alfalfa plants were inoculated with the Thy$^-$ or Thy$^+$ strains with and without the autoselective plasmid. At 80 days after sowing, 91% of the nodules from the Thy$^-$ mutant-inoculated plot still contained the autoselective plasmid, compared to 67% of the nodules from the Thy$^+$ strain. Nodules were found on the roots of plants inoculated with the Thy$^-$ strain which did not have the autoselective plasmid, presumably due to the reversion of the Thy$^-$ phenotype as all the isolates exhibited the chromosomal streptomycin resistance of the Thy$^-$ strain. In addition, plants from the uninoculated plot were not nodulated, demonstrating the absence of indigenous *R. meliloti* strains from the site.

Three years after release, the site was analyzed again to evaluate the survival of the released strains and plasmid maintenance. During the period between the initial evaluation and the subsequent evaluation, the site remained fallow and did not support a crop of alfalfa. The released rhizobia at the site had declined by several log numbers over the three-year period, but had become established at the site at low numbers. Examination of isolates revealed that plasmid stability in the plot which had received the Thy$^-$ strain containing the autoselective plasmid had declined from 91% to 55% (Fig. 4). An even more striking decline was observed for the plot which had received the Thy$^+$ strain containing the autoselective plasmid in which plasmid stability declined from 67% to 5% (Fig. 4). This work has demonstrated the effectiveness of thy-based containment systems in assuring stable plasmid maintenance in *R. meliloti* inoculant strains under relevant field conditions. These studies also indicated that spontaneous Thy mutants may not be suitable due to their ability to revert to the Thy$^+$ phenotype.

To overcome this obstacle, we have been developing a stable *thyA* mutant in *R. meliloti* which will not revert easily. Inactivation of the *thyA* gene in *R. meliloti* was achieved by insertion of a kanamycin-resistance gene cassette into this locus. The construct was subsequently introduced into *R. meliloti* strain F34 and replacement of the wild type *thyA* gene by the mutated construct by homologous recombination was selected for. Successful homologous recombination of the construct into the genome was detected by colonies which were kanamycin-resistant, gentamycin sensitive and Thy$^-$.

In combination with the development of a stable *thyA* mutant, we have been developing a system for the conditional expression of *thyA*. To achieve this objective, the *thyA* gene from strain F34 has been fused to the *nifH* promoter from *R. meliloti* which should allow for conditional expression of *thyA* under symbiotic (i.e. microaerobic) conditions. Expression of *thyA* should occur only under microaerobic conditions present in root nodules. Expression should not occur outside of the nodule resulting in the loss of viability of the inoculant. Introduction of a stable vector containing this construct into *E. coli* strain HX2, which is a Thy mutant resulted in complementation of the Thy

mutation. Subsequent curing of the plasmid restored the thymidine requirement. This fact demonstrates that the construct is capable of successful complementation of Thy mutants. Studies are currently underway to determine if this construct will complement a Thy mutation in *R. meliloti* under microaerobic conditions.

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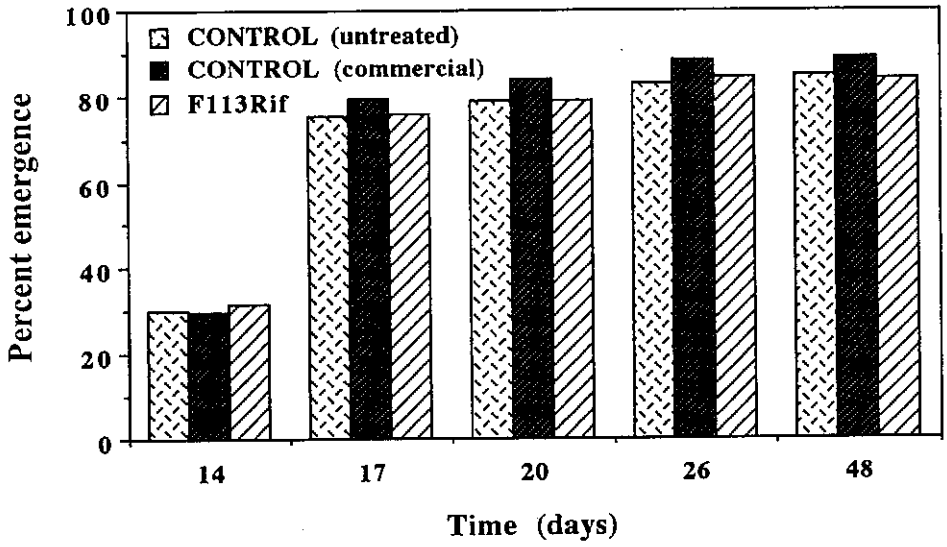

Fig.1 Emergence of sugarbeet at Bandon in 1994. Values are expressed as percent seeds germinated

The level of F113Rif on inoculated seeds was log 6.0 CFU/seed.

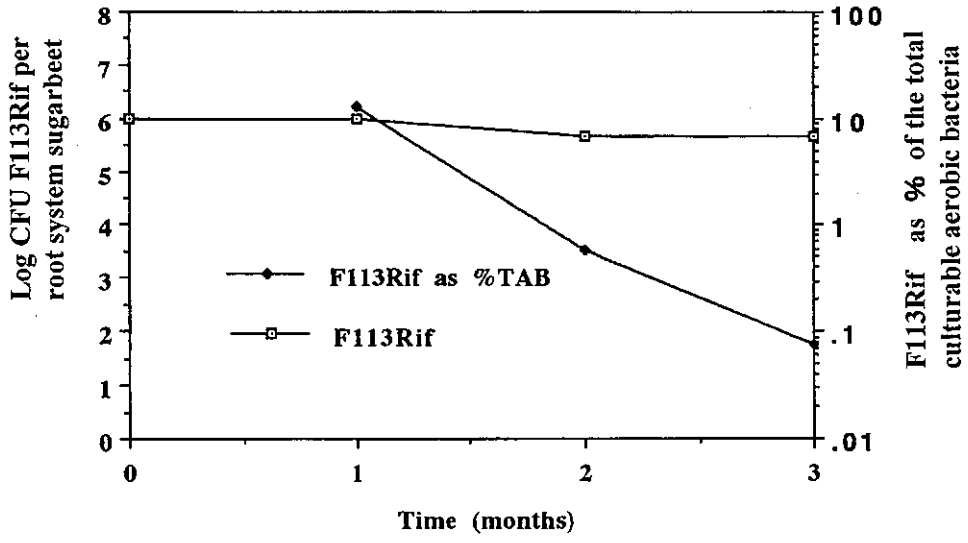

Fig.2 Colonization of sugarbeet inoculated with F113Rif at Bandon 1994 expressed as percentage of total aerobic bacteria (TAB)

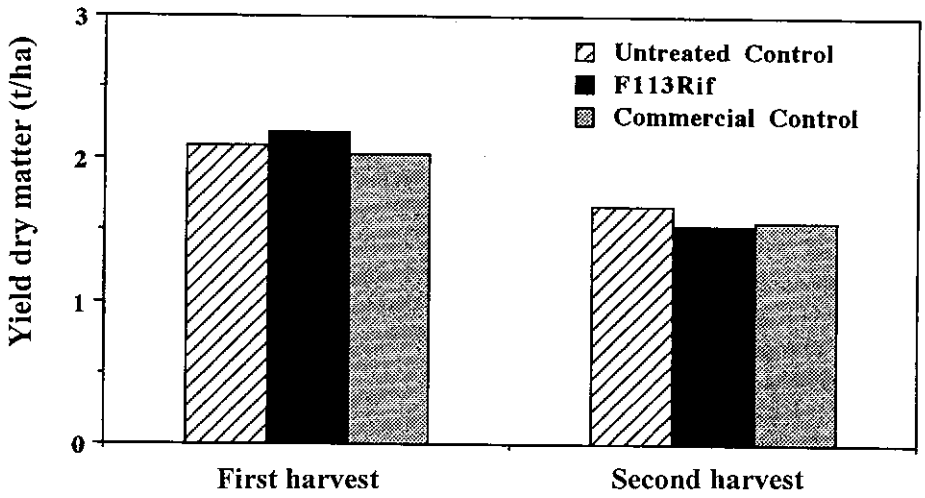

Fig.3 Impact of the biocontrol P. fluorescens F113 introduced as sugarbeet seed inoculant at Bandon in 1994 on yield of a red clover rotation crop in 1995

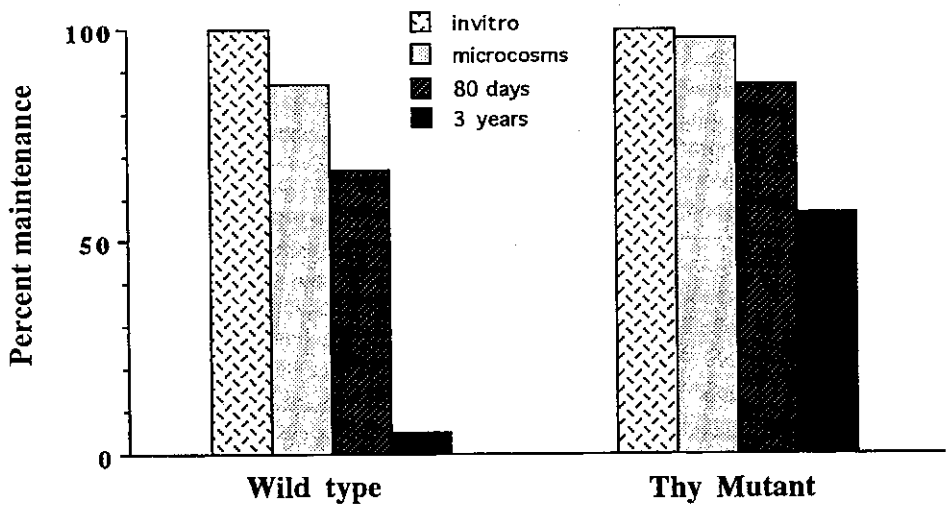

Fig.4 Maintenance of an autoselective thyA plasmid in *R. Idaho's* wild type and and Thy mutants.

Experimental conditions included in vitro, microcosm, and field release.