

Field Release of Genetically Modified Biofertilizers and Phytostimulators

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Abstract

During 1994-95 and 1995-96, genetically modified (GM) strains of *Rhizobium leguminosarum* bv. *viciae* and *Azospirillum brasilense* along with parental non-modified wild types were field-tested as biofertilizers and phytostimulators, respectively. Their fate was assessed, in terms of environmental impact, with respect to soil/rhizosphere microbiota and biochemical soil/plant activities. Determinations included soil microbial biomass, soil metabolic activities (respiration, N₂O emission), bacterial populations in the rhizosphere and soil, fluorescent pseudomonads, vesicular-arbuscular mycorrhizae. The experimental design of field tests allowed to compare the effects of bacterial strains with high and low colonizing ability along with strains containing the reporter genes as stable and unstable constructs. The performance consistency of the inoculants was also evaluated by means of statistical analysis across multiple field trials, including the assessment of effects on crops.

Introduction

In the framework of the research project IMPACT, supported by the European Commission, the environmental impact of different strains of biofertilizers (*Rhizobium leguminosarum* biovar *viciae*), phytostimulators (*Azospirillum brasilense*), and biocontrol agents (*Pseudomonas fluorescens*), released in open environments as genetically modified inoculants, was assessed during 1994-95 and 1995-96. An account of the results obtained during the field tests of 1994-95 has been reported by Corich *et al.* (1995) and Vamerali *et al.* (1995). Here we report the data obtained during the second cropping season, 1995-96. The data are comparatively evaluated with respect to the first release.

Microorganisms and their release

Several strains of *Rhizobium leguminosarum* biovar *viciae* were constructed.

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(Giacomini *et al.*, 1994; Corich *et al.*, 1996), which included the allochthonous wild type 1003 and the following derivatives: strain 1110 containing pDG3 with the reporter cartridge *lacZ-mer* (relatively stable plasmid), strain 1111 containing pDG4 with the same reporter cartridge (unstable plasmid, due to constitutive *lacZ* expression), and strain 1112 containing the cartridge inserted into the *recA* gene region of the chromosome (one copy, stable construct). Agri-10, a second *R. leguminosarum* biovar *viciae* was used as an autochthonous strain isolated from the soil site used for the release in 1995, along with the derived strain 1114, containing pDG3. *Azospirillum brasilense* strains Sp6 and Sp245 (wild types) were used along with their marked derivatives containing *gusA*, i.e. Sp6gusA normal producer of indole-3-acetic acid (IAA, 20-40 µg/l) and Sp6 IAA⁺⁺gusA producing 40% more IAA with respect to the parental strain (Vanderleyden *et al.*, 1996).

The releases were authorized by the national Competent Authority as required by Directive 220/90/EC and Italian DL 92 of 03/03/93. GM rhizobia were released at a site 12 km east of Padova, within the Experimental Station of the University of Padova (Agripolis, Legnaro), GM azospirilli were used at a site 4 km north of Ravenna. In both cases we used the same fields where the previous releases had been performed in 1994-95. The overall area of plots for field tests with azospirilli was 3000 m², while tests with rhizobia were carried out on a smaller scale, over a 50 m² area (Fig. 1 and Fig. 2). For the latter, the decision was made on a precautional basis, since unstable genetic material was to be used. Fences around the site and steel covers for plots during seed germination, were installed because of the presence of birds, mice and voles in the area (Fig. 1). This is a precautionary measure normally adopted also for neighboring experiments with non-modified organisms. For the release of GM and non-GM azospirilli with stable integration of marker genes, an empty aisle around the plots was left, hosting traps to reveal horizontal escapes of inoculated strains, possibly carried or enhanced by the water table movement.

Among the determinations reported in Table 1, we selected those parameters which were considered to be more reliable after microcosm or pre-release tests in soil, and easier to be performed on a routine basis.

Effects of GMMs on plants

As for *Rhizobium* inoculants, two situations have been compared in 1994 and 1995, i.e. strains derived from the allochthonous inoculant 1003 and strains derived from the autochthonous strain Agri-10. All the GM derivatives (1110, 1111, 1112 from 1003 and 1114 from Agri-10) were able to effectively nodulate the homologous hosts *Pisum sativum* and *Vicia faba minor*. However, the allochthonous strain and derivatives were found to be poor symbiotic performers, giving rise to a nodule occupancy of 3-7%, while the indigenous strain Agri-10 and its derivative were good performers on the same host plants. For Agri-10 and its GM-derivative, nodule occupancy ranged from 66-68% and 52-55%, respectively, suggesting that genetic modification may have affected the symbiotic performances of the inoculant.

As for *Azospirillum brasilense*, the GM strain Sp6IAA⁺⁺gusA was found to stimulate crop emergence of *Sorghum bicolor* (+ 3%, $p < 0.01$), crop yield (+9%, $p < 0.01$) and root length density (+44%, $p < 0.05$), with respect to the wild type. In Table 2 the values of root length density (RLD) are reported, at different soil depths.

These results enabled to confirm the effects on plant reported earlier for non-GM *A. brasilense* (Barbieri *et al.*, 1991), stressing the role of IAA produced by *Azospirillum*. It was interesting to note that the effects on RLD were more evident in the first 40 cm zone of the root canopy below the soil surface. This fact may suggest that the stimulatory activity of the plant-associated bacterium was higher soon after root formation and during the first phases of root elongation.

Effects of GMMs on microbial biomass and soil microbial activities

In Table 3 the results concerning soil microbial biomass, at harvest time of *Sorghum bicolor* inoculated with GM and non-GM *Azospirillum* strain, are listed. The values obtained for the various treatments were not significantly different, suggesting that variations, if any, remained below the least significant difference (ca. 15%) which was experimentally defined during pre-release tests (data not shown). From the values obtained, it appears that there is no significant impact of GM inoculants on microbial biomass. Significant differences among treatments could not be identified also for vesicular-arbuscular mycorrhizae formation on soybean roots, following inoculation with non-GM *Bradyrhizobium japonicum* and/or GM *Pseudomonas fluorescens* (Fig. 3-4). However, the semi-quantitative approach adopted, i.e. visual inspection and microscopy, needs further improvement to allow statistical evaluation of data. The use of recently developed DNA-based techniques (Stocchi *et al.*, 1995) might be helpful for future studies.

The production of gaseous nitrogen, essentially measured as N₂O emission from soil, was tested in a separate set of experiments during the cultivation of soybean plants inoculated with GM *Pseudomonas fluorescens* (Corich *et al.*, 1995) and/or non-GM *Bradyrhizobium japonicum*. A remarkable decrease of denitrification was observed in the presence of *Pseudomonas* during the field test in 1994 (Fig. 5A). In 1995 (Fig. 5B), the inhibition of denitrification due to the presence of GM *Pseudomonas* could not be confirmed. The discrepancy may be ascribed to the heavy rainfall during the first ten days after inoculation of GM and non-GM strains (75 mm of rain versus 30-35 mm, based on the average rainfall of the last 10 years). Our current interpretation of data is that a wash-out of the microbial cells could have occurred. The decrease might have concomitantly been enhanced by the increase of protozoan population feeding on microbial biomass, a phenomenon which has been repeatedly described in the literature.

Persistence of GMMs

One year after inoculation at the release sites, the GM strains were evaluated for their persistence. *Rhizobium leguminosarum* bv. *viciae* could be detected by using the reporter gene cartridge at a density of 10^2 - 10^3 cfu / gr of soil in all the plots where inoculated. In this case, a plate count assay of viable and culturable cells has been used, with a minimum detection limit of 100 cells/gr of soil. The method is rapid and results are obtained within 48 hours. Using the traditional MPN count method with plants (Vincent, 1982), the minimum detection limit can be lowered to ca. 1 cell/gr of soil, but the test lasts approximately 24 days.

After one year, *Azospirillum* was below detection limit, i.e. 10^2 cfu /gr soil. Whether this low concentration was affected by heavy rains as reported in the previous paragraph, remains to be elucidated. The same climatic factor could have affected the persistence of GM *Ps. fluorescens* which was present at 10^2 cfu/gr soil after one year, and below this minimum detection limit 505 days after inoculation.

Conclusive remarks

Clearly, the most appropriate methodological approach to define the environmental impact of released microbial inoculants, will be selected case by case. That will largely depend on (a) the microbial species and strain used in the field, (b) the need to reveal gross changes in the environment or fine-tuned dynamics, (c) the reproducibility and confidence limit of statistical evaluation of data, (d) the inherent properties of soil/environment where the release occurred, (e) the ease, time and effort which are deemed necessary for the analysis, also in terms of human resources and requirement for sophisticated equipment.

Based on our experience from the risk assessments conducted during the releases of GMMs in 1994-95 and 1995-96, it is suggested that among the available methods, the overall microbial activity in soil/water/plant ecosystems can be studied through CO₂ emission kinetics when gross changes (i.e. above 20%) are to be expected among treatments. If smaller differences are expected, gas-chromatography can be used instead of chemical analysis. However, the latter method is applicable on a much smaller scale with soil samples or in the field. Microbial biomass carbon is another very useful parameter to be measured, particularly if tests are repeated periodically. Under our conditions, the lowest significant difference was ca. 15% vs. control plots. A disadvantage of the method is that it is tedious and labor-intensive.

Gas-chromatographic determination of N₂O emission is more reliable to detect smaller environmental changes, although it is considered to be labor-intensive. Plate counts can be informative for selected bacterial populations, and quantitative monitoring of microfungi can rely upon rapidly evolving techniques (Stocchi *et al.*, 1995; Jensen *et al.*, 1996). However, the problems associated with viable and non-culturable microbial populations have not been solved yet, while microbial biodiversity appreciation has received increasing attention through non-cultural approaches such as scanning elec-

tron microscopy of microbiota examined *in situ* and analyzed by laser scanning confocal microscopy, digital image analysis, and biogeostatistics (Dazzo *et al.*, 1993).

Based on the results obtained from the release of GM biofertilizers and phytostimulators during 1994-95 and 1995-96, the following concluding remarks can be drawn:

- the behavior of GM *Rhizobium* strains in the field reflected what could be predicted on a laboratory scale and by microcosm experiments, at least in terms of stability of genetic information added to microbial strains;
- the use of catabolic marker genes (*lacZ*, *lacZY*) appears to be advantageous for routine identification of *Rhizobium* (and *Pseudomonas*) to be released as soil / seed inoculants;
- the use of the above markers appears to be “biosafe” in the short and medium term; data on long-term effects are not available;
- from a biosafety point of view GM population bursts or escapes from the release site remained below the detection limit of adopted techniques, i.e. 100 viable cells / gr of soil (d.w.);
- GM biofertilizers and phytostimulators can have a remarkable environmental impact, although the risk can be assessed as “very low” or “negligible”;
- differences between GM and non-GM strains often remained below the detection limit of the available techniques; studies on long-term effects of GMMs on the environment appear largely overlooked in literature.

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Table 1 Determinations useful for evaluating the environmental impact of GMMs used as inoculants in open environments

- microbial biomass
- number of viable m.o. (plate counts)
 - number of viable gram negative (plate counts)
 - number of viable non-symbiotic N₂-fixers
- bacterial populations in rhizosphere and rhizoplane
- fluorescent pseudomonads
- microfungi
- ectotrophic mycorrhizae and VAM
 - cellulolytic and ligninolytic
 - chitin degraders
 - denitrifiers and nitrifiers
 - nutritional groups (min. medium +/- growth factors)
 - energy source utilization
 - number of protozoa (predators)
- metabolic microbial activities (e.g. respiration, kinetics of CO₂ evolution)
- activity of soil enzymes
- kinetics of N transformation (NH₄⁺, NO₂⁻, NO₃⁻, N₂O emission)
 - biodiversity of microbial species (colony type, metabolic traits including antibiotic resistance)
- soil colonizing ability
- niche colonizing ability (e.g. nodule occupancy)
 - biomass of colonized plants
- biomass of plant shoots
- root hair and root length density (RLD)
 - NPK and micronutrient content of colonized plants

: parameters measured during releases of GM rhizobia and azospirilli in 1994-96

Table 2 Effects of GM *A. brasilense* on sorghum RLD at different nitrogen levels

Factor		RLD (cm of root / cm ³ soil) at soil depth (cm)		
Strain	Nitrogen (kg /ha)	0 - 40	40 - 100	0 -100
Control	0	1.77 b	0.66 a	1.13 c
Sp6gusA	0	1.93 b	0.70 a	1.21 bc
Sp6gusA	80	1.94 b	0.73 a	1.21 bc
Sp6IAA ⁺⁺	0	2.62 a	0.73 a	1.52 a
Sp6IAA ⁺⁺	80	2.48 a	0.78 a	1.38 ab

LSD test significance at $p < 0.05$. Survey at the end of elongation stage

Table 3 Soil microbial biomass 120 days after release of GM *A. brasilense*

Test	Carbon ($\mu\text{g} / \text{g}$)	Nitrogen ($\mu\text{g} / \text{g}$)	Biomass C/N	Biomass C / organic C
Control	279.00	32.32	8.64	3.18
Sp245lacZ	320.50	39.23	8.17	3.65
Sp6gusA	275.47	33.18	8.49	3.14
Sp6IAA ⁺⁺ gusA	296.42	33.53	8.89	3.38

Data represent the average of three replicates



Fig. 1 Site of release of GM and non-GM *Rhizobium leguminosarum* biovar *viciae* strains

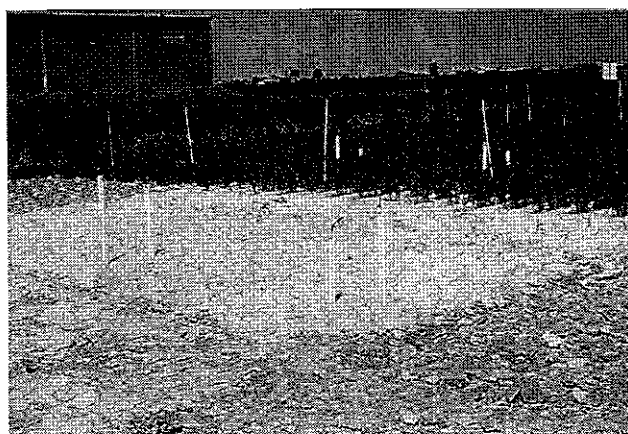


Fig. 2 Site of release of GM and non-GM *Azospirillum brasilense* strains

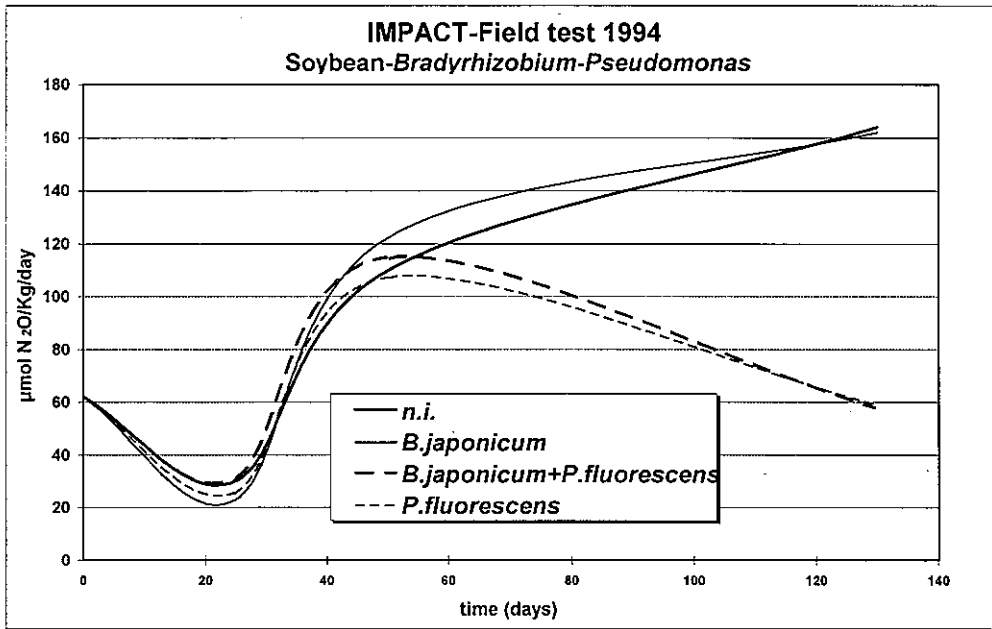


Fig. 3 Micrographs of thin sections of soybean roots showing VAM formation three weeks after inoculation with non-GM strains

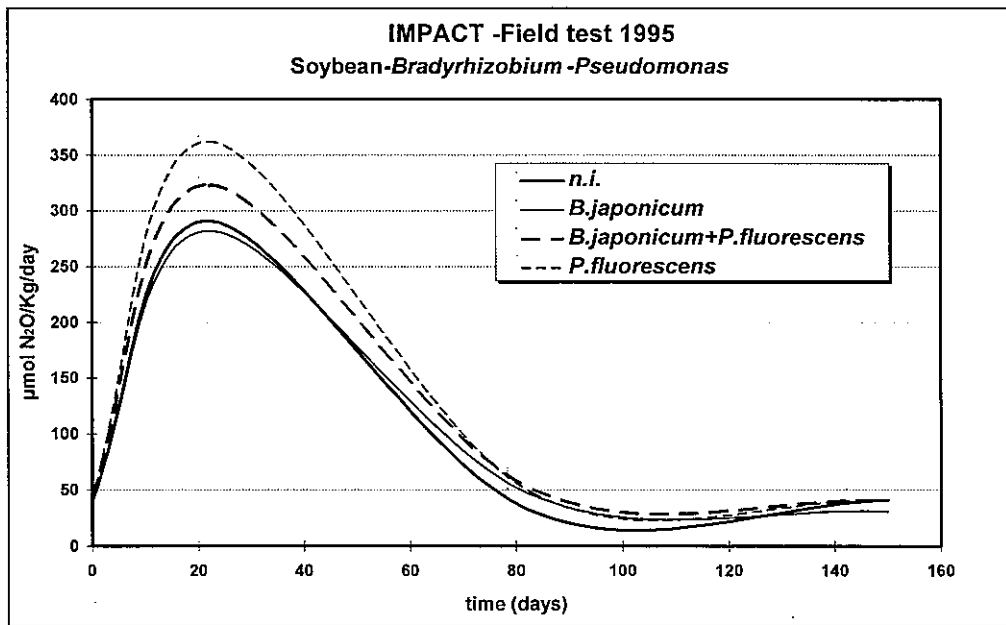
Upper side: uninoculated control plants; lower left: plants inoculated with *B. japonicum*; lower right: plants inoculated with *Ps. fluorescens*



Fig. 4 Micrographs of three thin sections of soybean roots showing VAM formation three weeks after co-inoculation with GM *Ps. fluorescens* and non-GM *B. japonicum*



A



B

Fig. 5 Denitrification (nitrous oxide emission) from soil cropped with soybean in 1994 (A) and 1995 (B)

Inoculation was performed with non-GM *B. japonicum* and GM *Ps. fluorescens* (*n.i.* = not inoculated).

