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Soil Biology & Biochemistry 38 (2006) 1712–1726

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Field survival of the phytostimulator *Azospirillum lipoferum* CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation

Hamdy El Zemrany[a,b], Jérôme Cortet[c,1], Matthias Peter Lutz[d], André Chabert[e], Ezékiel Baudoin[a,2], Jacqueline Haurat[a], Nicolas Maughan[c], Denis Félix[f], Geneviève Défago[d], René Bally[a], Yvan Moënne-Loccoz[a,*]

[a]*UMR CNRS 5557 Ecologie Microbienne, Université Claude Bernard (Lyon 1), 43 bd du 11 Novembre, 69622 Villeurbanne cedex, France*
[b]*Department of Soil Science, Faculty of Agriculture Shebin El-Kom, Minufiya University, Egypt*
[c]*UMR IMEP CNRS 6116, Europôle de l'Arbois, BP 80, 13545 Aix-en-Provence cedex 04, France*
[d]*Phytopathology Group, Institute of Plant Sciences, Swiss Federal Institute of Technology (ETH), CH-8092 Zürich, Switzerland*
[e]*Association de Coordination Technique Agricole (ACTA), La Saulsaie, 01120 Montluel, France*
[f]*Lycée Agricole de La Côte St André, F-38260 La Côte St André, France*

Received 13 April 2005; received in revised form 4 November 2005; accepted 9 November 2005
Available online 14 February 2006

Abstract

Heavy nitrogen fertilisation is often implemented in maize cropping systems, but it can have negative environmental effects. Nitrogen-fixing, phytohormone-producing *Azospirillum* plant growth-promoting rhizobacteria (PGPR) have been proposed as crop inoculants to maintain high yield when decreasing nitrogen fertilisation. In this context, agronomic and ecological effects of the inoculation of maize seeds with the PGPR *Azospirillum lipoferum* CRT1 were studied in two consecutive years. The inoculant was recovered from maize at 10^5 CFU g^{-1} root or higher. Inoculation enhanced root growth and development based on results of root biomass, rooting depth and/or parameters describing root system architecture, and a transient positive effect on shoot height was observed in the first year. Inoculation did not increase yield, but reducing mineral nitrogen fertilisation had only a minor effect on yield. This suggests that the lack of positive effect of the PGPR on yield was due to the fact that the whole field was heavily fertilised in years prior to the start of the experiment. Soil nitrogen levels decreased during the 2 years of the study, and the inoculant had no effect on residual soil nitrogen levels at harvest. Inoculation had no impact on *Fusarium* symptoms and concentration of the mycotoxin deoxynivalenol in maize kernels, but both were influenced by the interaction between inoculation and nitrogen fertilisation level. Inoculation did not influence meso/macrofaunal soil populations, but had a small but significant effect (smaller than the effect of added nitrogen) on decomposition, nitrogen mineralisation and mesofaunal colonisation of maize leaves (in litter bags). Overall, the ecological impact of seed inoculation with the PGPR *A. lipoferum* CRT1 was small, and its magnitude was smaller than that of chemical nitrogen fertilisation.

Keywords: *Azospirillum*; Phytostimulator; Rhizosphere; Ecological impact; Maize; *Fusarium*; Deoxynivalenol; Soil fauna; Litter bags

1. Introduction

Several *Azospirillum* strains have been studied as plant growth-promoting rhizobacteria (PGPR) (Bally et al., 1983; Okon and Labandera-Gonzalez, 1994; Jacoud et al., 1999; Dobbelaere et al., 2001). Their plant-beneficial effects result mostly from morphological and physiological changes of the root system, noticeably an increase in root proliferation and elongation rates (thus increasing the total

*Corresponding author. Tel.: +33 4 72 43 13 49; fax: +33 4 72 43 12 23.
E-mail address: moenne@biomserv.univ-lyon1.fr (Y. Moënne-Loccoz).
[1]Present address: Laboratoire Sols et Environment, UMR 1120 ENSAIA-INPL/INRA, 2 avenue de la forêt de Haye, 54505 Vandoeuvre-lès-Nancy, France.
[2]Present address: IRD, Centre IRD-ISRA, BP 1386, Dakar, Senegal.

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doi:10.1016/j.soilbio.2005.11.025

root surface area), which in turn leads to improved uptake of water and mineral nutrients (Okon and Kapulnik, 1986; Jacoud et al., 1999). These effects are mostly the consequence of phytohormone production (auxins, gibberellins and cytokinins) by *Azospirillum* (Dobbelaere et al., 1999), and they have been documented with different types of crops (often cereals) under different soil and climatic conditions (Okon, 1994; Dobbelaere et al., 2001). Plant growth-promoting effects of *Azospirillum* inoculants may lead to improved crop yield (Okon and Labandera-Gonzalez, 1994; Dobbelaere et al., 2001).

Bacteria of the genus *Azospirillum* are also free-living nitrogen fixers, but the contribution of nitrogen fixation to plant growth promotion seems less important than that of phytohormone production. In addition, phytohormone-mediated root proliferation effects of these bacteria can help the plant take up a larger part of the combined nitrogen present in soil (Okon and Kapulnik, 1986). Therefore, in addition to plant-growth promotion (Dobbelaere et al., 2001), inoculation of the crop with *Azospirillum* strains could also be useful to enable a reduction in mineral nitrogen input without compromising crop yield, in a context of low/lower input agriculture, and/or reduce pollution problems resulting from the use of large amounts of mineral nitrogen fertilisers. However, the ecological impact of this environmental biotechnology is little documented so far (Corich et al., 1995; Basaglia et al., 2003; Russo et al., 2005), and the objective of this work was to address this issue using a maize field heavily fertilised for more than 10 years (200 kg N ha^{-1} or more each year).

To reach this goal, field experiments in which the PGPR *Azospirillum lipoferum* CRT1 was inoculated to maize seed were carried out in 2001 and 2002 at La Côte Saint-André (Isère, France). CRT1 inoculation was studied at three levels of mineral nitrogen fertilisation to mimic a scenario of decreasing nitrogen fertilisation input (i.e. at two of the three N levels in 2001 and at all three N levels in 2002). The functional effect of the inoculant was assessed on maize crop. Furthermore, *Azospirillum* inoculation can lead to a significant modification of the architecture and functioning of the root system, which in turn may have an effect on plant shoot properties. Therefore, maize crop assessment also included an investigation of mycotoxigenic *Fusarium* spp., which infect plant shoots, produce mycotoxins on grains, and overwinter in crop residues. They can produce different types of mycotoxins, which may affect both animal and human consumers of the crop, and deoxynivalenol (DON) is one of the main mycotoxins produced by these fungi.

In addition, possible effects of *Azospirillum* inoculation (and of different nitrogen fertilisation levels) on plant composition may, in turn, have an impact on microbial decomposers and soil fauna feeding mostly on microbes and soil organic matter, which could affect the decomposition of crop residues. Furthermore, above-ground arthropod predators (spiders, etc.) depend in part on below-ground preys such as animal decomposers (see review by Scheu, 2001). These possibilities were assessed using litter bags, which are nylon bags filled with plant parts (here maize leaves) and placed onto the soil, enabling the entry and exit of the mesofauna (Crossley and Hoglund, 1962; Cortet and Poinsot-Balaguer, 1999; Knacker et al., 2003). The litter bag approach has been successfully used as a standard to assess disturbances to soil functioning (Cortet et al., 2002b; Römbke et al., 2002). In addition, litter bags were used to monitor the dynamics of mesofaunal populations, which were also studied in soil samples. Finally, the study was completed with an assessment of key soil macrofaunal groups, including earthworms, slugs and epigeic macroarthropods.

2. Materials and methods

2.1. PGPR strain and inoculum preparation

A. lipoferum CRT1 was isolated in France from the rhizosphere of field-grown maize (Fages and Mulard, 1988). This strain was chosen as inoculant because it is an effective PGPR (Fages and Mulard, 1988; Jacoud et al., 1998, 1999) and has undergone commercialisation as phytostimulator of maize (Lipha/Nitragin, Meyzieu, France). Maize seeds were mixed with CRT1 cells present in the commercial peat-based Azo-Green™ formulation (kindly supplied by Lipha/Nitragin, Meyzieu, France and Milwaukee, WI, USA) and distilled water: about 20,000 seeds were used for 400 g Azo-Green® containing about 5.5×10^{11} CRT1 cells. Therefore, inoculum level was approximately 2.8×10^7 CFU added per seed, which was confirmed by colony counts on modified Nitrogen-free (Nfb) agar (Nelson and Knowles, 1978) containing 0.2 g l^{-1} ammonium chloride and Congo Red (Rodriguez Caceres, 1982).

2.2. Field experiment

The experiment was conducted in 2001 and 2002 at a field site (45°22′43″ and 5°16′02″) located 50 km South-East of Lyon in La Côte Saint-André (Isère, France) (Russo et al., 2005). The site corresponds to a flat glacier valley and the soil is a luvisol (FAO), syn. alfisol (US Soil Taxonomy). Soil characteristics in the loamy surface horizon (0–20 cm) were clay 16.2%, fine silt 27.5%, coarse silt 16.4%, fine sand 16.2%, coarse sand 23.8%, organic matter 2.1%, pH (water) 7.0, CEC 8.2 meq $(100\,g)^{-1}$, N 1.4 g kg^{-1}, P 0.62 g kg^{-1}, K 0.31 g kg^{-1}, Ca 1.9 g kg^{-1}, C/N ratio 8.0. The field was cropped with maize for more than 5 years prior to the experiment. Bovine-ovine compost (organic carbon 219 g kg^{-1}, total nitrogen 18.3 g kg^{-1}, C/N ratio 12.0, phosphorus 10.6 g kg^{-1}, potassium 53.3 g kg^{-1}, calcium 86.9 g kg^{-1}, magnesium 5.2 g kg^{-1}, pH 9.0) was applied to the whole field in February 2001 (5 t ha^{-1}; bringing 92 kg N ha^{-1}), but was not used in 2002. It is estimated that the 2001 compost application provided 55 and 18 kg mineral N ha^{-1} during 2001 and 2002, respectively, based on previous agronomic experimentation at the

site and weather conditions in both years. In both years, soil preparation was done in February (tillage, 20–25 cm depth) and April (harrowing).

The maize sowing season ranges from late April to late May in the area. In 2001, maize seeds (*Zea mays* 'Eurostar'; Rustica, Mondonville, France) were sown on May 29, as heavy rainfall prevented earlier sowing. In 2002, sowing was done on April 25, using an earlier cultivar ('PR38a24'; Pioneer, Aussonne, France). In 2001, seeds were treated with the fungicides fludioxonil at 25 mg kg^{-1} seed, metalaxil at 20 mg kg^{-1} seed and anthraquinone at 900 mg kg^{-1}, whereas chemical seed fungicides were not used in 2002. Sowing was done at 80,000 seeds ha^{-1} in both years.

In 2001, the herbicides Atraphyt (1.6 l ha^{-1}, i.e. atrazine, 0.8 kg ha^{-1}) and Frontière (1.3 l ha^{-1}, i.e. dimethenamide, 1.2 kg ha^{-1}) were applied on May 8, and Banvel 4S (0.6 l ha^{-1}, i.e. dicamba, 0.29 kg ha^{-1}) on June 22 (between rows). In 2002, the herbicides IFT Acajou (0.6 l ha^{-1}, i.e. isoxaflutol, 45 g ha^{-1} and aclonifen, 0.3 kg ha^{-1}), Isard (0.8 l ha^{-1}, i.e. Dmta-p, 0.58 kg ha^{-1}) and Atraphyt (1.5 l ha^{-1}, i.e. atrazine, 0.75 kg ha^{-1}) were applied on April 27, and Banvel 4S on May 20 (0.4 l ha^{-1}, i.e. dicamba, 0.19 kg ha^{-1}) and June 8 (0.2 l ha^{-1}, i.e. dicamba, 96 g ha^{-1}).

2.3. Experimental treatments

In 2001, the effect of inoculation with *A. lipoferum* CRT1 was studied at each of three levels of mineral nitrogen fertilisation (i.e. 0, 70 and 130 kg N ha^{-1}), using a factorial design with six combinations of factors (i.e. inoculation or no inoculation × three mineral N levels; Experiment 1). The rate of 130 kg mineral N ha^{-1} in combination with compost (i.e. a total estimated 185 kg N ha^{-1} available during the 2001 growing season) is comparable to (i) fertilisation levels used prior to the experiment and (ii) current recommendations made to farmers in the area (190–200 N ha^{-1}). Experimental plots were 6 m wide (i.e. 8 rows) × 15 m long. They were organised along a randomised block design with four blocks (i.e. 24 plots; Fig. 1). Mineral nitrogen fertiliser consisted of half-nitrate and half-ammonium and was applied on 5 June (50 kg N ha^{-1} for fertilised plots) and 26 June (20 and 80 kg N ha^{-1} for the plots at 70 and 130 kg N ha^{-1}, respectively).

In 2002, compost was not added. The treatments used in 2001 (i.e. inoculation or no inoculation × three mineral N levels) were applied again to the same plots in Experiment 1. This time, even the highest N level corresponded to reduced fertilisation input. In addition, a smaller experiment (Experiment 2; Fig. 1) was started with eight new plots, which in 2001 had been grown with non-inoculated maize receiving 100 kg mineral N ha^{-1}. These eight plots were 3 m wide (i.e. 4 rows) × 15 m long. In 2002, they received 70 kg mineral N ha^{-1} and half the plots were sown with inoculated seeds, the other half being sown with non-inoculated seeds. All nitrogen doses were applied on June 10 in both experiments.

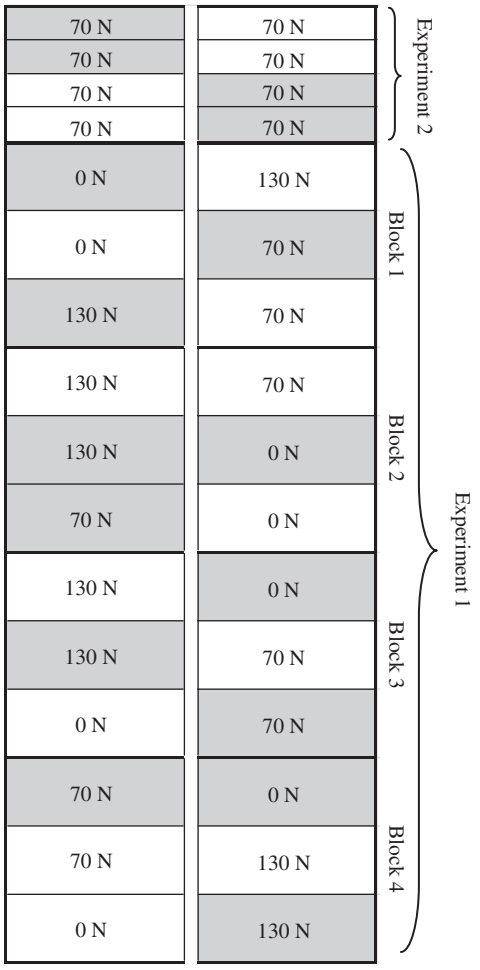

Fig. 1. Experimental design at La Côte Saint André for Experiments 1 (2001 and 2002) and 2 (2002). Mineral nitrogen fertilisation levels (0, 70 and 130 kg N ha^{-1} yr^{-1}) are referred to as 0, 70 and 130 N. Grey boxes refer to CRT1-inoculated plots.

2.4. Agronomic analysis of the crop

For root system analysis, maize plants were sampled at four dates in 2001 and three dates in 2002. In 2001, maize samplings were done at 7 (on June 5; at 2–3 leaves), 35 (on July 3; at 9–10 leaves), 65 (on August 2) and 142 d (on October 17) after sowing. The three 2002 samplings were done also at 2–3 (on May 13; at 18 d) and 9–10 leaves (on June 21; at 57 d), to facilitate comparisons between the 2 years, as well as at 112 d (on August 16). Within each plot studied, plants were chosen at random along one inner row for a given sampling time and their whole root systems were unearthed.

Plant growth was monitored based on the biomass and size of roots and shoots. In 2001, only plots receiving 70 kg mineral N ha^{-1} were studied at the first three samplings, whereas all plots were studied at the fourth sampling (Experiment 1). In 2002, plant growth was assessed as in 2001. Only plots receiving 70 kg mineral N ha^{-1} were studied at the first two samplings, whereas all plots were studied at the third sampling (Experiments 1 and 2). In

2002, root architecture was also investigated by image analysis (WinRHIZO; Régent Instruments Inc., Québec City, Qué., Canada) at 2–3 leaves, by quantifying the total root length, the root surface area, the average root diameter, the total root volume and the number of roots. Each analysis was carried out using two plants per plot. Hydro N-Tester measurements, which are directly related to chlorophyll levels in leaves, were carried out in each plot ($n = 30$) shortly before flowering, on August 8, 2001 and July 30, 2002.

Yield was assessed using Experiment 1. A silage-type harvest, i.e. harvest of whole shoots for silage (which is used as cattle feed in the region) was done on September 20, 2001 and September 12, 2002. Whole shoots were taken over about 10 m of one inner row in each plot. Grain harvest (October 17, 2001 and September 19, 2002) was carried out by sampling cobs of plants from another inner row in each plot. At both types of harvests, nitrogen content (assessed using 300 g of composite plant sample) and biomass analyses were done in each plot, in collaboration with the Institut du Végétal (ARVALIS; Paris, France).

2.5. Monitoring of inoculant survival on roots

Monitoring of *A. lipoferum* CRT1 on roots was carried out at each root sampling for plots receiving 70 kg mineral N ha^{-1}. Three root systems were used per plot at each sampling and they were independently processed, immediately upon arrival in the lab. Roots were vigorously shaken to get rid of loosely adhering soil and dipped in sterile distilled water for 5 min. They were cut in 1-cm pieces and ground for 2 min in phosphate buffer (NaCl 8.5 g, $NaH_2PO_4 \cdot 12H_2O$ 0.4 g, $Na_2HPO_4 \cdot 12H_2O$ 2.5 g, distilled water 1 l). In both years, fresh root material amounted to 0.10 g (in 20 ml buffer) at the first sampling and 8.0 g (in 100 ml) at the second and grinding was done using an Ultrax (Janke & Kunkel Labortechnik, Staufen, Germany) at medium speed. In the first year, fresh root material amounted to 170 g (in 300 ml buffer) at the third sampling and 100 g (in 300 ml buffer) at the fourth, and a Waring blender 8011EB (New Hartford, CT, USA) was used. Dilution series were prepared in phosphate buffer and plating was done in triplicate onto Nfb agar containing 0.2 $g\,l^{-1}$ ammonium chloride and Congo Red. *Azospirillum*-like colonies were identified based on colony morphology (mucous, scarlet pink colonies) after 5 d of growth at 28 °C.

Azospirillum-like colonies were further studied by colony hybridisation using a 16S rDNA-targeted oligonucleotide (Kabir et al., 1994; Chotte et al., 2002) for *A. lipoferum* (probe Al: 5′-CGTCGGATTAGGTAGT-3′; hybridisation/washing temperature of 43 °C), as described by Chotte et al. (2002) with minor modifications. Probe synthesis was done by PROLIGO (Paris, France). Briefly, colonies were grown in duplicate for 48 h at 28 °C on sterile membranes (GeneScreen Plus; Nen Life Science Products, Boston, MA, USA) placed on tryptone-yeast extract plates (Beringer, 1974). For cell lysis, the membranes were treated successively for 6 min in 10% (w/v) sodium dodecyl sulphate (SDS), 10 min in denaturing solution (NaOH 0.5 M and NaCl 1.5 M), 9 min in neutralising solution (NaCl 1.5 M, Tris 1 M, pH 7.4) and 9 min in 2 × standard saline citrate (SSC; NaCl 0.1 M, sodium citrate 15 mM, pH 7.0). After being left drying for 1 h on 3 MM paper, the membranes were exposed to UV for 4 min for DNA binding. For hybridisation, the membranes were moistened in 2 × SSC, rolled and transferred into hybridisation tubes containing pre-hybridisation solution (16.5 ml sterile distilled water, 3 ml dextran 50%, 1.5 ml SDS 10%, 0.58 g NaCl). The tubes were placed 2 h in an oven set at 65 °C before adding the probe (5′-end-labelled with ^{32}P, according to Sambrook et al., 1989) and 600 µl of denatured herring sperm DNA. After a 12-h hybridisation, the membranes were rinsed twice with 2 × SSC (5 min, at room temperature), twice with 2 × SSC and 1% SDS (30 min) and three times with 0.1 × SSC (30 min). The membranes were sealed in plastic bags and stored at −80 °C prior to autoradiography. Colonies reacting to the *A. lipoferum* probe were subjected to a second hybridisation (hybridisation/washing temperature of 65 °C) to identify *A. lipoferum* CRT1 using a strain-specific probe (accession number U90627) described by Jacoud et al. (1998).

2.6. Analysis of mycotoxinogenic Fusarium spp. colonising maize

Mycotoxinogenic *Fusarium* spp. colonising maize were studied in 2002. For isolation of *Fusarium* species, maize residues (stems and roots) from the 2001 growing season were collected on 29 April 2002 to have an overview of *Fusarium* spp. present in the experimental field. The stems were cut longitudinally and the roots into pieces. Samples showing *Fusarium* symptoms were cut out, surface-sterilised for 45 s in a 7% (vol/vol) sodium hypochlorite solution (Erne-Chemie, Avenches, Switzerland) and washed twice with sterile water in a sterile hood. These samples were incubated on malt extract (1.5%) agar plates amended with rifampicine (100 µg ml^{-1}) to avoid bacterial contamination. After 7 d at 24 °C in the dark, the fungi were identified based on morphological properties using an optical microscope.

Prior to the 2002 grain harvest, 20 cobs (rachis) were randomly hand-picked from each of the 24 plots in Experiment 1 and husked. Stems (first internodium) were also sampled but visible symptoms of *Fusarium* infection were not found. Maize kernels showing *Fusarium* symptoms were treated as explained above to identify the *Fusarium* species causing ear blight. In addition, disease assessment and DON analyses were carried out on these cobs. Disease severity was evaluated using a 7-class rating scale where 1 = no infection, 2 = 1–3%, 3 = 4–10%, 4 = 11–25%, 5 = 26–50%, 6 = 51–75%, and 7 = more than 75% of the kernels exhibit visible symptoms of ear rot, such as pinkish mycelial growth (Reid and Hamilton,

1996). After assessing symptoms, 10 ears were selected randomly from each plot and the kernels were removed by hand from each rachis. The kernels were mixed thoroughly to obtain a random distribution of the kernels and a 500-g sample was dried for 24 h at 60 °C. The samples were ground to a fine powder in an Ultra Centrifugal Mill type ZM1 (Retsch GmbH & Co., Haan, Germany) with a 1 mm wire mesh. From all samples, a 1.5 g sub-sample was taken to quantify DON levels using a commercial ELISA method (R-Biopharm, Darmstadt, Germany).

2.7. Analysis of fauna in soil and colonising maize litter

Analysis of soil fauna was carried out in 2002, using Experiment 1. Soil macrofauna was sampled in plots receiving 70 kg mineral N ha^{-1}, as follows. The *Carabidae*, *Staphylinidae* and *Arachnidae* (spiders) families were trapped using 500-cm^3 plastic pots (containing 250 ml water with detergent) buried up to the brim (five pots per plot) on May 2. The content of each pot was then weekly collected over a 2-month period and analysed using morphological criteria (Holland, 2002). Earthworms were collected on November 28 and 29 by sprinkling formalin over the soil surface (25 ml of 30% formalin solution for a 1 m^2 patch and after the first sprinkling a second with 50 ml; two patches per plot), and their biomass was determined (Lee, 1985). Slugs were collected in the same way and counted.

Both soil cores and litter bags (Cortet et al., 2002a, b) were used to collect microarthropods (i.e. mesofauna) in each plot, as follows. At each sampling date (May 13, June 21 and September 5), three soil cores (5 cm diameter, 10 cm depth) taken within a central row were collected per plot, making 216 soil cores in total. Litter bags consisted of 5 g of dried (45 °C, 48 h) maize leaves in 12 × 12 cm Nylon bags (4 mm mesh size). The maize leaves introduced in the bags had been collected on the same field plots on 17 October 2001 (i.e. at grain harvest). Thus, for each 2002 treatment, the bags used contained maize that had received the same treatment the previous year. Nine bags were placed 30 cm apart on the bare soil surface of each plot (between the third and fourth rows) on May 13 (i.e. 18 d after sowing), which made 216 bags in total. Three bags were sampled per plot 39 (June 21), 115 (September 5) and 150 (October 10) days later. The soil cores and litter bags sampled were put in plastic bags and brought to the laboratory for processing.

Microarthropods were extracted from soil cores and litter bags using Berlèse funnels (over 10 d) and kept in 70% alcohol (Berlèse, 1905). Collembola and mites were identified based on morphological features, as described by Gisin (1960) for Collembola and Krantz (1978) for mites, and the corresponding individuals counted.

2.8. Analysis of maize litter biodegradation

Litter bags were also used to monitor litter biodegradation, as follows. Litter mass remaining (LMR) and N concentration of the litter-bag samples were derived from calibration equations using near-infrared reflectance spectrophotometry (NIRS), a methodology highly suitable for litter decomposition analysis (Cortet et al., 2003). For that purpose, remaining litter samples were carefully cleaned of soil particles (by sieving; 4 mm mesh), dried (24 h at 45 °C) and milled with a Cyclotec 1093 (1 mm mesh size). All 216 samples were scanned using a NIRS-system 6500 spectrophotometer (Foss Nirs Systems, Inc., Silver Spring, MD, USA). Two replicate reflectance measurements of monochromatic light were made at 2 nm intervals over the 400–2500 nm range to produce an average spectrum with 1050 data points. Reflectance (R) was converted to absorbance (A) using the following equation: $A = \log(1/R)$. Maize residue components from our samples were predicted using calibration equations previously determined with another spectral and chemical data set. This spectral and chemical database used to build the calibration equations related to the ash and nitrogen concentration for each sample was comprised of 720 maize residue samples collected in La Côte Saint André fields (Cortet et al., 2002b). The concentrations of these maize components in these calibration set samples were determined using wet chemistry methods: ash residues were determined from 48 samples (550 °C for 3 h) and nitrogen concentration from 49 samples (Kjeldahl method). Calibration equations between spectral and chemical data were conducted using the ISI software system (Shenk and Westerhaus, 1991). The ash-free LMR was then calculated for each sample using the following equation:

$$\mathrm{LMR}_i = 100 \times \frac{\mathrm{MD}_i}{\mathrm{MD}_0} \frac{100 - \mathrm{AT}_i}{100 - \mathrm{AT}_0},$$

where LMR_i is the percentage of remaining litter mass of sample i; MD_i the dry mass of sample i; MD_0 the dry mass of initial sample i; AT_i the % total ash concentration of sample i; AT_0 the % initial total ash concentration of sample i.

From estimated N and ash content of each sample, N concentration of the organic matter was calculated for each sample:

$$T_i = 100 \times \frac{\mathrm{NC}_i}{100 - \mathrm{AT}_i},$$

where T_i is the % N concentration in organic matter for sample i; NC_i the N concentration in sample i.

2.9. Statistical analysis

Root-colonising population data for *A. lipoferum* CRT1 were expressed per gram of dry root and log-transformed. For microarthropod numbers, raw data (x) were transformed as $\log(2x+1)$. At each sampling, data were subjected to analysis of variance using StatView 4.5 (Microsoft Corporation, Paris, France) and means were compared when appropriate using Fisher's or Bonferroni's tests. One-way analysis of variance served (i) to compare

CFU from both experiments at each sampling in 2002, and (ii) assess the effect of inoculation when only plots at 70 kg N ha^{-1} were studied (i.e. macrofaunal data and certain plant data). Two-way (*Azospirillum* inoculation × nitrogen fertilisation) analysis of variance was used for other plant data, *Fusarium* symptoms and DON levels, LMR and N concentration of maize litter, and microarthropod data. Correlation analysis was done using Pearson coefficient or Spearman rank correlation coefficient, and Bonferroni probability. All analyses were done at $P<0.05$ level.

3. Results

3.1. Survival of inoculated A. lipoferum CRT1 on maize roots

Colonisation of maize roots by *A. lipoferum* CRT1 was monitored in plots receiving 70 kg mineral N ha^{-1}. In 2001, the inoculant was recovered at high levels (around 8 log CFU g^{-1} root) at 7 (2–3 leaves) and 35 d (9 leaves), and at lower numbers at subsequent samplings (e.g. less than 6 log CFU g^{-1} root at 142 d) (Fig. 2). In 2002, the inoculant was recovered at levels (around 8 log CFU g^{-1} root) similar to those found in 2001 in the same plots at the same stages of plant development (2–3 and 9 leaves). Similar results were obtained in the second experiment started in 2002 (Fig. 2).

3.2. Effect of inoculation with A. lipoferum CRT1 on maize growth

In 2001 (Experiment 1), inoculation with *A. lipoferum* CRT1 had a positive effect on rooting depth (at each sampling) and root system biomass (from day 35 on) in plots receiving 70 kg N ha^{-1} (Table 1). The beneficial effect on root biomass in these plots was also observed at the time of grain harvest (Table 2). A comparable positive effect of the inoculant on root biomass at harvest was found in plots receiving 130 kg N ha^{-1}, but not in plots that did not receive mineral nitrogen. At 70 kg N ha^{-1}, a modest

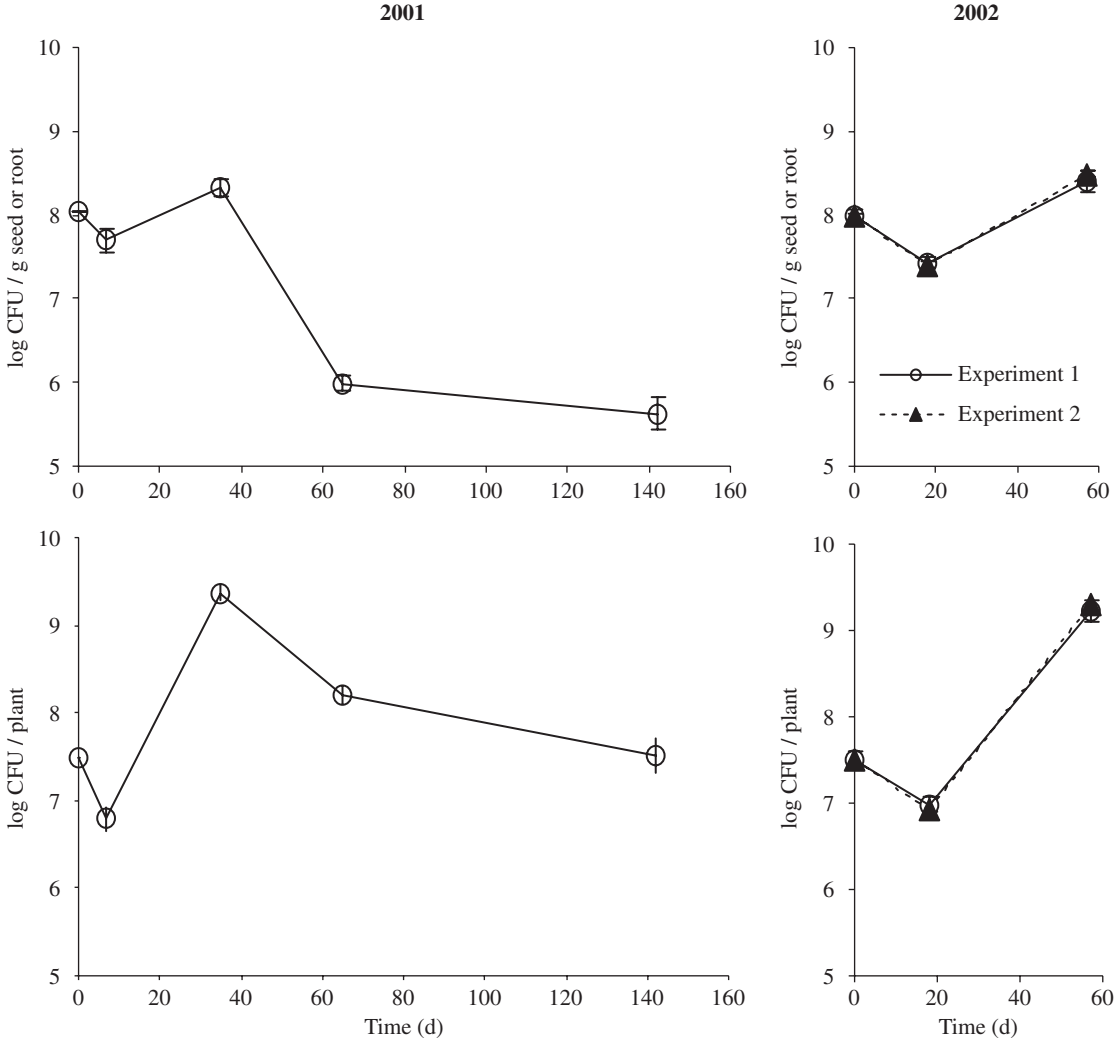

Fig. 2. Survival of the inoculant *A. lipoferum* CRT1 on maize roots in 2001 and 2002. CFU (mean ± SE; $n = 12$) are expressed per root system or per gram of root. The growth stages studied in 2002, i.e. 2–3 leaves (at 18 d) and 9 leaves (at 57 d) were the same as the ones for the first two 2001 samplings (i.e. 7 and 35 d). In 2002, there was no significant difference in CFU at any of the samplings.

Table 1
Effect of *A. lipoferum* CRT1 on roots and shoots at different samplings in 2001 for plots in Experiment 1 receiving 70 kg N ha^{-1} (mean ± SE; $n = 4$ plots[a])

Parameters	Day 7		Day 35		Day 65	
	Control	Inoculated	Control	Inoculated	Control	Inoculated
Root weight (g $plant^{-1}$)	0.12 ± 0.01	0.14 ± 0.02	7.6 ± 0.6 a[b]	11.4 ± 0.7 b	107 ± 8 a	173 ± 12 b
Rooting depth (cm)	6.5 ± 0.7 a	8.8 ± 0.8 b	12.7 ± 0.4 a	17.9 ± 0.8 b	22.7 ± 0.3 a	28.2 ± 0.8 b
Shoot height (cm)	8.9 ± 0.2	9.3 ± 0.4	69.3 ± 1.7 a	87.5 ± 1.2 b	252 ± 7	250 ± 3

[a]For each treatment, three plants were studied in each of the four plots at each sampling.
[b]Statistical differences between treatments are indicated by letters a and b at each sampling ($P < 0.05$).

Table 2
Effect of *A. lipoferum* CRT1 on root biomass prior to grain harvest in Experiment 1 (mean ± SE; $n = 4$ plots[a])

Root parameters	0 kg N ha^{-1}		70 kg N ha^{-1}		130 kg N ha^{-1}	
	Control	Inoculated	Control	Inoculated	Control	Inoculated
2001						
Brace roots (g $plant^{-1}$)	19.8 ± 1.6 a[b]	23.4 ± 0.7 a	18.9 ± 0.9 a	36.7 ± 1.6 b	21.2 ± 0.6 a	31.3 ± 2.0 b
Other roots (g $plant^{-1}$)	68.7 ± 1.9 ac	70.7 ± 2.8 ac	59.8 ± 1.7 a	113.6 ± 2.5 b	75.7 ± 2.3 c	119.2 ± 5.7 b
Total roots (g $plant^{-1}$)	88.5 ± 2.0 ac	94.1 ± 3.5 ac	78.6 ± 2.2 a	150.3 ± 2.7 b	96.9 ± 2.4 c	150.5 ± 4.5 b
% Brace roots among total roots	22.0 ± 1.5	25.1 ± 0.2	23.8 ± 1.0	24.4 ± 1.0	22.2 ± 2.4	22.2 ± 1.7
2002						
Brace roots (g $plant^{-1}$)	11.9 ± 1.1	10.4 ± 1.1	16.2 ± 1.1	19.7 ± 1.6	13.3 ± 1.2	16.8 ± 1.2
Other roots (g $plant^{-1}$)	23.1 ± 1.3	27.6 ± 2.9	42.9 ± 1.3	27.3 ± 2.5	36.6 ± 2.2	34.2 ± 2.7
Total roots (g $plant^{-1}$)	35.0 ± 2.1	38.3 ± 3.1	59.1 ± 2.1	47.8 ± 2.7	49.8 ± 2.2	50.9 ± 2.9
% Brace roots among total roots	31.9 ± 2.3	32.7 ± 2.5	27.2 ± 2.3	39.8 ± 1.0	27.2 ± 2.2	35.3 ± 2.3

[a]For each treatment, three plants were studied in each of the four plots at each sampling.
[b]Statistical differences between treatments are indicated with letters a, b and c ($P < 0.05$).

Table 3
Effect of *A. lipoferum* CRT1 on roots and shoots at different samplings in 2002 for plots receiving 70 kg N ha^{-1} (mean ± SE; $n = 4$ plots[a])

Parameters	Day 18[b]		Day 57	
	Control	Inoculated	Control	Inoculated
Experiment 1				
Root weight (g $plant^{-1}$)	0.27 ± 0.02 a[c]	0.38 ± 0.03 b	5.6 ± 0.6	6.8 ± 0.3
Rooting depth (cm)	6.4 ± 0.4 a	9.5 ± 0.7 b	16.2 ± 0.7 a	22.8 ± 1.1 b
Shoot height (cm)	8.3 ± 0.3	8.7 ± 0.3	65.2 ± 2.7	69.8 ± 1.4
Experiment 2				
Root weight (g $plant^{-1}$)	0.26 ± 0.01 a	0.34 ± 0.02 b	5.3 ± 0.5	6.8 ± 0.5
Rooting depth (cm)	6.3 ± 0.6 a	8.8 ± 0.7 b	14.3 ± 0.8 a	22.8 ± 0.9 b
Shoot height (cm)	7.8 ± 0.3	8.5 ± 0.3	66.1 ± 3.4	66.5 ± 1.7

[a]For each treatment, three plants were studied in each of the four plots at each sampling.
[b]Plants were at 2–3 leaves at 18 d and 9 leaves at 57 d, i.e. the same growth stages as the two first 2001 samplings.
[c]Statistical differences between treatments are indicated by letters a and b at each sampling ($P < 0.05$).

but statistically significant effect of CRT1 on shoot height was seen at 35 d, but not at 7 or 65 d (Table 1).

In 2002, the positive effect of *A. lipoferum* CRT1 on root system biomass in plots receiving 70 kg N ha^{-1} took place again in Experiment 1, but it disappeared by day 57 (Table 3). In contrast, the positive effect on rooting depth was significant at both 18 and 57 d. Similar results were obtained in Experiment 2. In both experiments, the inoculation treatment had no effect on root biomass at 112 d (Table 2), regardless of N fertilisation level (for Experiment 1). The small positive effect of CRT1 on shoot height seen in 2001 in Experiment 1 was not found in 2002 in any of the two experiments.

In 2002, a more detailed analysis of the effect of CRT1 on root development was carried out at 18 d, using image analysis (Table 4). Results indicated that CRT1 inoculation could have positive effects on the number of roots per plant, the total root length per plant, the total root volume

Table 4
Effect of *A. lipoferum* CRT1 on root system architecture at the first 2002 sampling (18 d), as characterised by image analysis of root system parameters (mean ± SE; $n = 8$[a])

Root system parameters	Experiment 1						Experiment 2	
	0 kg N ha^{-1}		70 kg N ha^{-1}		130 kg N ha^{-1}		70 kg N ha^{-1}	
	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated
Total root length (cm plant^{-1})	33.4 ± 3.4 a[b]	46.1 ± 4.8 b	40 ± 4.6	44.2 ± 2.8	32.2 ± 3.3 a	42.6 ± 5 b	40.2 ± 4.4	47.8 ± 4.8
Root surface area (cm^2 plant^{-1})	12.9 ± 0.8 a	17.3 ± 1.5 b	15.3 ± 1.4	16.3 ± 1.0	12.3 ± 1.2	15.5 ± 1.3	14.7 ± 1.2	16.8 ± 1.0
Average root diameter (mm)	1.28 ± 0.09	1.21 ± 0.04	1.26 ± 0.06	1.17 ± 0.03	1.22 ± 0.05	1.19 ± 0.04	1.19 ± 0.06	1.16 ± 0.07
Total root volume (cm^3 plant^{-1})	0.41 ± 0.03 a	0.52 ± 0.04 b	0.47 ± 0.03	0.48 ± 0.04	0.38 ± 0.04 a	0.46 ± 0.03 b	0.43 ± 0.03 a	0.48 ± 0.02 b
Number of roots	144 ± 22 a	218 ± 37 b	159 ± 34	195 ± 27	116 ± 22 a	187 ± 42 b	217 ± 33	206 ± 39

[a]For each treatment, two plants were studied in each of the four plots at each sampling.
[b]Statistical differences between control and inoculated treatments (at each N level in Experiment 1) are indicated by letters a and b ($P < 0.05$).

and the total root surface area, but the average root diameter was not modified by inoculation (as the trend for a lower average root diameter in inoculated plants was not significant at $P < 0.05$). The positive effect of CRT1 on root development depended on the rate of mineral nitrogen fertilisation. Noticeably, the effect of the inoculant was statistically significant at 0 and 130 kg N ha^{-1} but essentially not at 70 kg N ha^{-1} (Table 4), despite a significant effect on rooting depth and root biomass (Table 3).

3.3. Effect of inoculation with *A. lipoferum* CRT1 on mycotoxinogenic *Fusarium spp.*

A total of 33 *Fusarium* isolates were obtained from maize stems collected in spring 2002. About 66% of these isolates were determined microscopically as *F. graminearum* and the remaining 44% as *F. equiseti*. Nineteen isolates of *Fusarium* sp. were found on roots. In contrast to the isolates derived from stems, only 21% of the root isolates belonged to *F. graminearum*, whereas 79% were *F. equiseti*. No other *Fusarium* species was found. Only *F. graminearum* was evidenced on infected kernels sampled at the time of grain harvest (data not shown).

No stem infection was found at the time of sampling. Disease severity of the maize cobs were low, as they ranged between 1.89 and 2.65. The distribution of disease symptoms within plots was rather patchy. Nitrogen fertilisation had no significant influence on disease severity when *Azospirillum* was not used. However, when maize was inoculated, the addition of nitrogen fertiliser had a small but statistically significant effect on disease severity. Indeed, the application of 70 kg N ha^{-1} (but not 130 kg N ha^{-1}) reduced disease severity significantly in inoculated maize (Table 5).

DON contamination of kernels ranged between 0.9 and 2.7 μg g^{-1} (dry weights) (Table 5). The levels of DON were not influenced by N fertilisation in the absence of inoculation. In inoculated maize, DON contamination was 65% lower at 70 kg N ha^{-1} compared with no fertilisation. DON concentration in maize can be directly related to the extent of *Fusarium* contamination (Reid and Sinha, 1998), and therefore this possibility was assessed here. A positive correlation was found between disease severity and DON contamination level when analysing together the mean data obtained for the six treatments in Experiment 1 and the two treatments in Experiment 2 (Pearson correlation coefficient $r = 0.927$, Bonferroni probability level $P = 0.001$; $n = 8$).

3.4. Effect of inoculation with *A. lipoferum* CRT1 on agronomic crop parameters at harvests

The positive effects of the inoculant on root parameters did not translate into any agronomic effect on above-ground parts at silage and grain harvests in Experiment 1, as indicated by analysis of the 10 parameters studied in 2001 and 2002 (Table 6). The effect of nitrogen fertilisation was significant for some of the parameters studied (e.g. grain yield and grain nitrogen content) but not all (e.g. ear density, N-Tester). When so, results at 70 and 130 kg N ha^{-1} did not differ, but differed from those obtained in the absence of nitrogen fertilisation.

3.5. Effect of inoculation with *A. lipoferum* CRT1 on mineral nitrogen levels in soil

The impact of CRT1 inoculation on soil levels of mineral nitrogen was assessed in 2001 and 2002, at three soil depths. Data indicate that inoculation had no effect on residual nitrate and ammonium levels present in soil at the time of grain harvest (Table 7). The effect of nitrogen fertilisation at harvest was significant for nitrate in 2002 (but not in 2001), at 0–30 and 30–60 cm.

3.6. Effect of inoculation with *A. lipoferum* CRT1 on soil fauna

The impact of inoculation with *A. lipoferum* CRT1 on soil macrofauna was assessed in 2002, at plots receiving 70 kg N ha^{-1}. Results indicated that inoculation had no significant effect on any of the macrofaunal groups investigated (Table 8).

Table 5
Effect of *A. lipoferum* CRT1 on *Fusarium* ear rot symptoms (disease severity) and contamination of kernels with the mycotoxin deoxynivalenol (DON) in 2002 (mean ± SE; $n = 4$ plots)

Root system parameters	Experiment 1						Experiment 2	
	0 kg N ha^{-1}		70 kg N ha^{-1}		130 kg N ha^{-1}		70 kg N ha^{-1}	
	Control	Inoculated	Control	Inoculated	Control	Inoculated	Control	Inoculated
Disease severity[a]	2.20 ± 0.17 ab[c]	2.65 ± 0.15 a	2.08 ± 0.10 ab	1.89 ± 0.20 b	2.22 ± 0.14 ab	2.13 ± 0.27 ab	2.02 ± 0.22	1.94 ± 0.15
DON contamination[b] (μg g^{-1})	2.28 ± 0.31 ab	2.67 ± 0.34 a	1.43 ± 0.44 ab	0.92 ± 0.37 b	1.81 ± 0.63 ab	1.60 ± 0.62 ab	1.48 ± 0.39	1.25 ± 0.38

[a]Disease severity ratings are on a 1–7 scale ranging from no infection (1) to >75% of the kernels visually moldy (7). Each of the four values was derived from the analysis of 20 maize cobs.
[b]Each of the four values was derived from the analysis of 10 maize cobs.
[c]Within each experiment, statistical differences between treatments are indicated by letters a and b ($P < 0.05$).

Table 6
Effect of *A. lipoferum* CRT1 on the crop at silage and grain harvests in Experiment 1 (mean ± SE; $n = 4$ plots)

Crop parameters	0 kg N ha^{-1}		70 kg N ha^{-1}		130 kg N ha^{-1}		
	Control	Inoculated	Control	Inoculated	Control	Inoculated	
2001							
Plant density (× 1000 ha^{-1})	75.6 ± 0.4	75.6 ± 3.0	77.8 ± 0.8	73.4 ± 2.2	76.2 ± 1.4	75.6 ± 0.7	
Lodging (%)	11.1 ± 3.1	8.7 ± 2.7	14.8 ± 3.6	16.0 ± 3.0	16.0 ± 6.8	19.0 ± 4.5	
N-Tester[a]	637 ± 7	594 ± 24	771 ± 12	758 ± 15	794 ± 11	774 ± 8	
Shoot N content (g kg^{-1})	10.2 ± 0.6 c[b]	9.9 ± 0.2 c	12.0 ± 0.8 ab	11.8 ± 0.4 b	13.0 ± 0.5 ab	13.6 ± 0.7 a	N[c]
Shoot dry content (%)	34.5 ± 0.7	33.5 ± 0.6	33.0 ± 0.7	33.8 ± 0.8	33.8 ± 0.7	32.1 ± 0.6	
Shoot yield (t ha^{-1})	13.4 ± 1.4 c	13.7 ± 1.0 bc	16.8 ± 0.4 a	16.6 ± 0.6 a	18.2 ± 0.7 a	16.0 ± 0.5 ab	N
Ear density (× 1000 ha^{-1})	71.6 ± 0.8	72.2 ± 1.7	75.6 ± 0.6	72.8 ± 2.1	75.0 ± 1.4	74.4 ± 0.6	
Weight of 1000 grains (g)	282 ± 8	295 ± 8	322 ± 2	317 ± 9	330 ± 7	334 ± 4	
Grain yield (q ha^{-1})	79.8 ± 3.2 b	83.6 ± 4.2 b	111.4 ± 3.6 a	102.0 ± 3.3 a	107.6 ± 3.2 a	109.4 ± 2.4 a	N
Grain N content (g kg^{-1})	12.8 ± 0.2 b	13.3 ± 0.2 b	15.0 ± 0.5 a	14.7 ± 0.3 a	15.4 ± 0.3 a	15.2 ± 0.5 a	N
2002							
Plant density (× 1000 ha^{-1})	81.9 ± 1.7	82.1 ± 2.6	81.2 ± 1.7	80.8 ± 0.8	79.4 ± 1.5	77.2 ± 1.7	
Lodging (%)	8.6 ± 4.0	6.9 ± 2.0	8.5 ± 2.6	4.6 ± 2.2	6.6 ± 2.0	3.7 ± 1.5	
N-Tester	520 ± 13	611 ± 52	793 ± 17	652 ± 53	786 ± 24	801 ± 16	
Shoot N content (g kg^{-1})	4.1 ± 0.8	4.7 ± 0.4	5.4 ± 1.1	4.7 ± 0.8	5.6 ± 1.4	6.7 ± 1.0	
Shoot dry content (%)	23.4 ± 1.4	23.2 ± 1.4	23 ± 1.9	24.1 ± 0.7	23.2 ± 1.3	22.6 ± 1.6	
Shoot yield (t ha^{-1})	5.6 ± 0.1 c	6.0 ± 0.3 bc	7.1 ± 0.8 ab	6.9 ± 0.3 ab	8.0 ± 0.4 a	7.3 ± 0.2 ab	N
Ear density (× 1000 ha$^{-1)}$	80.0 ± 1.7	80.8 ± 2.6	80.3 ± 1.7	80.0 ± 0.8	78.7 ± 1.5	75.9 ± 1.7	
Weight of 1000 grains (g)	269 ± 7 d	287 ± 8 cd	316 ± 5 ab	301 ± 12 bc	328 ± 2 a	326 ± 11 ab	N
Grain yield (q ha^{-1})	54.7 ± 3.9 d	67.4 ± 5.9 cd	94.1 ± 2.2 ab	79.6 ± 10 bc	101.4 ± 2.2 a	99.0 ± 5.3 a	N
Grain N content (g kg^{-1})	8.9 ± 0.3 c	9.9 ± 0.8 c	11.6 ± 0.4 ab	10.1 ± 0.6 bc	12.0 ± 0.2 a	11.9 ± 0.3 a	N

[a]Light transmittance measurement of the ear leaf (correlates with leaf chlorophyll content).
[b]Statistical differences between treatments are indicated with letters a, b, c and d ($P < 0.05$).
[c]N indicates that the effect of nitrogen fertilisation was statistically significant.

Despite the higher root necromass left to decompose throughout inoculated plots at the higher N levels, no significant effect of *Azospirillum* inoculation (or nitrogen fertilisation) was found when analysing microarthropod data obtained in 2002 from soil cores (Table 9). In a few instances, the colonisation of litter bags by microarthropods was reduced when maize was inoculated (Table 9), noticeably in the case of the 'other microarthropods' in June (57 d after sowing) in plots at 70 kg N ha^{-1}, as well as the total microarthropods in September (133 d after sowing) in plots at 130 kg N ha^{-1}. However, nitrogen fertilisation had a greater effect than *Azospirillum* inoculation on colonisation of litter bags by microarthropods, in several cases with a trend for higher numbers at the intermediate fertilisation level (70 kg N ha^{-1}).

3.7. Effect of inoculation with *A. lipoferum* CRT1 on litter biodegradation

Decomposition of maize residues within litter bags was fast, as at 168 d after sowing LMR represented only 6–17% of the initial dry mass (Table 10). *Azospirillum* inoculation resulted in reduced LMR at 168 d under 0 kg N ha^{-1} (but not at 70 or 130 kg N ha^{-1}), but did not have any

Table 7
Soil contents in mineral nitrogen (kg N ha^{-1}) at the start and end of the 2001 and 2002 growing seasons in Experiment 1 (mean ± SE, $n = 4$ plots[a])

Sampling and nitrogen form	Soil depth (cm)	0 kg N ha^{-1}		70 kg N ha^{-1}		130 kg N ha^{-1}		
		Control	Inoculated	Control	Inoculated	Control	Inoculated	
2001 Sowing								
Nitrate	0–30	30.5 ± 4.7 b[b]	18.0 ± 0.8 a	29.5 ± 0.5 b	25.2 ± 3.4 ab	29.5 ± 3.1 b	24.0 ± 2.7 ab	I[c]
	30–60	23.2 ± 4.5	16.5 ± 1.7	20.0 ± 2.6	16.0 ± 2.1	19.2 ± 2.3	17.5 ± 2.0	
	60–90	16.5 ± 2.4	16.2 ± 1.9	13.7 ± 2.3	13.2 ± 1.1	16.0 ± 2.8	20.5 ± 6.1	
Ammonium	0–30	18.0 ± 3.7	20.0 ± 5.9	21.0 ± 5.0	21.5 ± 4.1	12.7 ± 1.9	15.5 ± 4.1	
	30–60	10.5 ± 2.1	9.0 ± 2.0	9.5 ± 1.8	7.0 ± 1.5	6.0 ± 0.4	8.7 ± 0.9	
	60–90	7.2 ± 1.5	4.0 ± 0.4	7.0 ± 1.1	8.5 ± 1.7	6.2 ± 1.7	8.0 ± 1.2	
2001 Grain harvest								
Nitrate	0–30	23.7 ± 4.8	12.5 ± 1.9	10.7 ± 1.7	12.2 ± 1.8	7.2 ± 0.5	7.7 ± 0.9	
	30–60	9.0 ± 1.1	8.7 ± 1.8	8.0 ± 0.7	8.2 ± 2.3	6.0 ± 0.4	5.5 ± 0.3	
	60–90	9.3 ± 1.1	8.2 ± 1.3	10.0 ± 2.1	8.5 ± 2.2	6.0 ± 0.6	5.7 ± 0.5	
Ammonium	0–30	6.2 ± 0.8	5.5 ± 1.0	7.0 ± 1.2	6.7 ± 1.1	6.5 ± 0.7	6.0 ± 0.7	
	30–60	5.2 ± 0.8	4.7 ± 0.5	6.5 ± 0.7	5.5 ± 0.7	6.2 ± 0.5	6.0 ± 0.4	
	60–90	5.7 ± 0.5	4.2 ± 0.7	7.0 ± 1.1	5.2 ± 0.8	5.7 ± 0.3	6.2 ± 0.5	
2002 Sowing								
Nitrate	0–30	7.2 ± 0.5	8.7 ± 1.5	7.5 ± 0.3	7.0 ± 0.4	7.7 ± 0.3	8.0 ± 0.4	
	30–60	7.0 ± 0.4	7.7 ± 0.5	7.7 ± 0.5	7.0 ± 0.4	7.5 ± 0.9	7.0 ± 0.4	
	60–90	7.7 ± 0.6	8.0 ± 0.0	8.2 ± 0.5	7.7 ± 0.5	7.5 ± 0.3	8.7 ± 0.3	
Ammonium	0–30	5.5 ± 1.3	7.0 ± 0.6	4.7 ± 1.1	5.0 ± 1.1	6.5 ± 0.9	6.0 ± 0.4	
	30–60	5.0 ± 0.7	6.2 ± 0.3	5.7 ± 1.3	6.0 ± 0.4	6.0 ± 0.4	5.2 ± 0.8	
	60–90	4.0 ± 0.4	4.5 ± 0.5	3.7 ± 0.3	4.0 ± 0.4	4.5 ± 0.5	5.0 ± 0.8	
2002 Grain harvest								
Nitrate	0–30	8.7 ± 0.9 b	9.0 ± 0.7 b	8.7 ± 1.1 b	8.3 ± 0.6 b	10.2 ± 0.8 ab	12.5 ± 1.4 a	N
	30–60	8.5 ± 0.7 ab	8.7 ± 0.6 ab	7.7 ± 0.5 ab	7.3 ± 0.3 a	10.2 ± 1.2 bc	11.5 ± 1.3 c	N
	60–90	9.5 ± 0.3	8.7 ± 0.3	9.2 ± 0.5	9.0 ± 0.4	10.2 ± 0.9	10.7 ± 1.1	
Ammonium	0–30	8.5 ± 1.3	7.3 ± 0.5	7.0 ± 1.1	7.7 ± 0.6	7.7 ± 0.5	5.7 ± 1.3	
	30–60	7.5 ± 0.9	8.0 ± 0.7	7.5 ± 0.7	7.3 ± 0.5	7.5 ± 0.7	7.5 ± 0.7	
	60–90	6.7 ± 0.9 ab	6.7 ± 0.5 ab	6.7 ± 0.9 ab	9.3 ± 1.7 a	5.5 ± 0.3 b	5.2 ± 0.5 b	N

[a]In each plot and at each depth, data were derived from chemical analysis of one composite sample consisting of 10 subsamples.
[b]Statistical differences between the six treatments are indicated with letters a and b ($P < 0.05$).
[c]I and N indicate, respectively, that the effect of inoculation (the effect seen at the 2001 sowing only reflects soil heterogeneity) and/or nitrogen fertilisation was statistically significant.

Table 8
Effects of *A. lipoferum* CRT1 on soil macrofauna in 2002 for plots in Experiment 1 receiving 70 kg N ha^{-1} (mean ± SE; $n = 4$ plots[a])

	Control	Inoculated[b]
Carabidae (Individuals plot^{-1})[c]	506 ± 172	442 ± 117
Staphylynidae (Individuals plot^{-1})	26.8 ± 8.5	39.5 ± 12.1
Arachnidae (Individuals plot^{-1})	91.3 ± 27.8	75.5 ± 22.1
Epigeic macroarthropods (total) (Individuals plot^{-1})	624 ± 197	557 ± 143
Slugs (Individuals m^{-2})	9.8 ± 7.9	6.0 ± 3.7
Earthworm biomass (g m^{-2})	21.1 ± 7.4	15.1 ± 3.0

[a]For each plot, data were derived from analysis of five traps or two 1-m^2 soil surface.
[b]Differences between inoculation and control were not statistically significant.
[c]Individuals trapped after 8 weeks of pitfall trap exposure (total for five traps).

significant effect on decomposition at the other samplings. In contrast, the addition of mineral nitrogen had a significant effect on litter decomposition at each sampling date, with higher N fertilisation levels resulting in reduced LMR. The relation between litter N content and LMR was studied using the N content of leaves sampled at the 2001 grain harvest (Table 10), as N data at silage harvest (Table 6) were obtained from whole, green shoots. Results showed that a significant negative correlation existed between the initial N content of maize residues in litter bags and the final LMR (Spearman correlation coefficient $r = -0.532$, $P < 0.0001$; $n = 71$).

Table 9
Effects of *A. lipoferum* CRT1 on numbers of microarthropods in soil cores or litter bags in 2002 in Experiment 1 (mean ± SE; $n = 4$ plots[a])

Taxon	Day[b]	0 kg N ha^{-1}		70 kg N ha^{-1}		130 kg N ha^{-1}		
		Control	Inoculated	Control	Inoculated	Control	Inoculated	
Soil cores								
Mites	18	7.42 ± 1.53	5.92 ± 1.25	4.83 ± 0.52	5.17 ± 0.82	4.58 ± ± 1.41	5.33 ± 1.03	
	57	12.8 ± 2.5	13.0 ± 2.0	11.8 ± 1.9	9.33 ± 1.39	7.83 ± 1.01	8.83 ± 1.64	
	133	13.8 ± 2.4	13.9 ± 1.9	7.75 ± 1.44	9.00 ± 1.43	11.2 ± 2.0	10.6 ± 1.3	
Collembola	18	3.50 ± 0.88	4.67 ± 1.00	8.08 ± 3.60	9.00 ± 4.08	6.08 ± 1.64	11.7 ± 7.4	
	57	0	0	0.08 ± 0.08	0.08 ± 0.08	0	0	
	133	12.6 ± 3.4	12.5 ± 2.3	11.2 ± 2.6	9.00 ± 1.88	11.1 ± 2.0	12.9 ± 1.7	
Other microarthropods	18	0.58 ± 0.29	1.25 ± 0.57	2.17 ± 0.61	1.00 ± 0.25	1.00 ± 0.39	0.92 ± 0.23	
	57	0	0.17 ± 0.11	0.17 ± 0.11	0.08 ± 0.08	0.25 ± 0.13	0.17 ± 0.11	
	133	1.50 ± 0.45	2.00 ± 0.62	1.42 ± 0.51	1.08 ± 0.26	1.50 ± 0.34	2.00 ± 0.49	
Microarthropods (total)	18	11.5 ± 1.9	11.8 ± 2.0	15.1 ± 3.8	15.2 ± 4.4	11.7 ± 2.1	17.9 ± 7.4	
	57	12.8 ± 2.5	13.2 ± 2.1	12.0 ± 1.9	9.5 ± 1.4	8.1 ± 1.1	9.0 ± 1.6	
	133	27.8 ± 4.5	28.4 ± 3.5	20.3 ± 4.0	19.1 ± 2.3	23.8 ± 3.1	25.5 ± 2.9	
Litter bags								
Mites	57	13.4 ± 2.4 a[c]	8.08 ± 1.22 ab	23.0 ± 9.0 a	26.3 ± 4.5 a	5.67 ± 2.19 b	11.8 ± 3.3 ab	N, I*N[d]
	133	67.7 ± 11.2 b	72.8 ± 18.2 b	89.3 ± 12.7 ab	92.9 ± 25.3 ab	137.3 ± 11.9 a	83.9 ± 14.2 ab	N
	168	115.8 ± 11.9	133.3 ± 13.7	158.6 ± 21.5	132.8 ± 20.2	164.3 ± 30.4	141.8 ± 22.4	
Collembola	57	0.08 ± 0.08	0.08 ± 0.08	0.17 ± 0.17	0	0	0	
	133	40.7 ± 4.5 a	27.3 ± 5.6 ab	42.0 ± 4.1 a	33.6 ± 4.0 a	24.8 ± 6.3 ab	13.6 ± 2.5 b	N, I
	168	30.8 ± 4.3 ab	35.5 ± 6.2 ab	60.5 ± 13.6 a	48.6 ± 10.8 a	25.3 ± 8.6 b	23.9 ± 5.4 b	N
Other microarthropods	57	1.08 ± 0.38 ab	1.17 ± 0.59 ab	3.67 ± 1.00 a	0.67 ± 0.36 b	2.00 ± 0.69 ab	0.92 ± 0.51 b	I
	133	2.08 ± 0.43	0.67 ± 0.23	1.17 ± 0.27	1.08 ± 0.36	1.00 ± 0.44	2.25 ± 1.45	
	168	1.67 ± 0.50	1.58 ± 0.43	1.33 ± 0.60	0.83 ± 0.21	0.50 ± 0.23	0.75 ± 0.25	
Microarthropods (total)	57	14.6 ± 2.4 ab	9.33 ± 1.14 ab	26.8 ± 9.2 a	26.9 ± 4.6 a	7.67 ± 2.29 b	12.7 ± 3.4 ab	N
	133	110.4 ± 13.6 ab	100.8 ± 19.2 b	132.5 ± 13.7 ab	127.6 ± 25.5 ab	163.1 ± 14.0 a	99.8 ± 14.1 b	I
	168	148.3 ± 11.8	170.4 ± 12.6	220.4 ± 18.6	182.2 ± 16.3	190.2 ± 30.8	166.4 ± 26.4	

[a]Data were derived from destructive analysis of 12 soil cores or 12 litter bags per plot at each sampling.
[b]Number of days after maize sowing (litter bags were placed on the soil surface 18 d after sowing), corresponding to 39, 115 and 150 d after placing litter bags in situ.
[c]Statistical differences between treatments are indicated with letters a and b ($P < 0.05$; Two-way ANOVA, with Bonferroni post hoc test).
[d]N and I indicate, respectively, that the effect of nitrogen fertilisation and/or inoculation was statistically significant. N*I indicates a significant interaction between the two factors.

Table 10
Effects of *A. lipoferum* CRT1 on maize litter mass remaining (LMR) and nitrogen concentration (*T*) of maize litter in 2002 in Experiment 1 (mean ± SE; $n = 4$ plots[a])

Parameter		0 kg N ha^{-1}		70 kg N ha^{-1}		130 kg N ha^{-1}		
	Day[b]	Control	Inoculated	Control	Inoculated	Control	Inoculated	
LMR (%)	57	65.5 ± 3.1 ab[c]	67.9 ± 2.6 a	56.8 ± 5.2 bc	55.6 ± 4.5 c	46.8 ± 7.5 d	52.4 ± 5.4 cd	N[d]
	133	16.7 ± 2.1 a	15.6 ± 1.4 ab	12.4 ± 2.5 cd	13.1 ± 1.8 bc	9.9 ± 1.3 d	9.4 ± 1.6 d	N
	168	17.0 ± 2.5 a	12.5 ± 1.1 b	9.7 ± 1.6 cd	11.3 ± 2.2 bc	6.3 ± 1.0 d	8.2 ± 1.8 d	N, I*N
T (%)	18	0.55[e]	0.43	0.55	0.48	0.55	0.67	
	57	0.85 ± 0.09 b[c]	0.83 ± 0.07 b	1.09 ± 0.10 a	1.04 ± 0.09 ab	1.21 ± 0.16 a	1.22 ± 0.18 a	N
	133	0.67 ± 0.03 c	0.70 ± 0.04 bc	0.86 ± 0.04 a	0.74 ± 0.03 b	0.83 ± 0.04 a	0.83 ± 0.03 a	I, N, I*N
	168	0.73 ± 0.03 b	0.71 ± 0.04 b	0.85 ± 0.04 a	0.76 ± 0.03 b	0.90 ± 0.05 a	0.83 ± 0.07 a	I, N, I*N

[a]Data were derived from NIRS analysis of three litter bags per plot at each sampling.
[b]Number of days after maize sowing (litter bags were placed on the soil surface 18 d after sowing), corresponding to 39, 115 and 150 d after placing litter bags in situ.
[c]Statistical differences between treatments are indicated with letters a, b, c and d ($P < 0.05$; Two-way ANOVA, with Bonferroni post hoc test).
[d]N and I indicate that the effect of, respectively, nitrogen fertilisation and inoculation was statistically significant; I*N indicates a significant interaction between the two factors.
[e]N content at the start of the 2002 litter-bag experiment, which was launched at 18 d after sowing, was determined on one composite sample of roughly chopped dried leaves collected at the 2001 grain harvest.

At 70 kg added N ha^{-1}, nitrogen concentrations of litter residues were significantly greater in non-inoculated plots compared with inoculated plots, both at 133 and 168 d after sowing (Table 10). The same trend was observed at 0 and 130 kg N ha^{-1}, but differences were not significant at $P<0.05$. The addition of mineral nitrogen had also a significant effect, with higher N levels resulting in higher litter N concentrations at each of the three samplings.

4. Discussion

Inoculant survival is a prerequisite for successful implementation of beneficial effects on the plant. Survival of inoculated *Azospirillum* varies according to soil, climatic and plant conditions (Bashan et al., 1995; Okon and Kapulnik, 1986). Here, *A. lipoferum* CRT1 survived well on maize roots in both years, especially during the first 2 months after inoculation. It means that at least from this viewpoint the ecological conditions were suitable to expect a positive effect on plant growth. Seeds inoculated with strain CRT1 were used 2 years consecutively in the same plots, but CRT1 colony count data obtained: (i) from bulk soil prior to the 2002 sowing in Experiment 1 (not shown) and (ii) from roots of inoculated maize in 2002 in both experiments (Fig. 2) suggest that recolonisation of the 2002 crop from soil by CRT1 cells introduced as seed inoculant in 2001 was unlikely to contribute significantly to the size of the CRT1 population on maize roots.

Several research groups have assessed the ecological impact of biocontrol inoculants, based on the rationale that these PGPR often produce antimicrobial compounds and are likely to interfere with the resident microbiota associated with roots (Moënne-Loccoz et al., 2001; Whipps, 2001). The possibility of direct negative effects of *Azospirillum* inoculants on resident microorganisms cannot be ruled out since: (i) competition of a bacterial inoculant with related, indigenous bacteria may take place (Moënne-Loccoz et al., 2001) and (ii) certain *Azospirillum* strains have the potential to control bacterial phytopathogens (Bashan and De-Bashan, 2002). However, *Azospirillum* PGPR are essentially documented as phytostimulators. As such, they may be expected to affect the resident microbiota mostly indirectly, i.e. via their effect on the plant and the rhizosphere environment.

The consequences of *Azospirillum* inoculation on chemical properties of the rhizosphere as microbial habitat are not always well understood. *A. brasilense* can induce acidification of the rhizosphere (Carrillo et al., 2002) and inoculation with phytohormone-producing *Azospirillum* may change root physiology and patterns of root exudation (Heulin et al., 1987). These features are difficult to assess under field conditions and were not studied in the current work. In addition, inoculation with *Azospirillum* typically results in a change in root architecture and an increase in root branching (Okon and Vanderleyden, 1997; Jacoud et al., 1999). Indeed, the use of *A. lipoferum* CRT1 had positive effects on root biomass and rooting depth in the current work (Tables 1–3). These effects were observed in both experiments, at different samplings, and (for Experiment 1) in both years. The effect of strain CRT1 on root biomass at the 2001 grain harvest was as high as +92% at 70 kg N ha^{-1}, which means that an additional 5 t of maize root were left in soil to decompose after harvest.

However, the positive effects of the inoculant on the root system did not result into a positive effect on above-ground parts in agronomic terms (Table 6), except for a small, transient increase of shoot height at 35 d in 2001 at 70 kg N ha^{-1} (Table 3) and greener leaves observed on 27 August 2002 at 0 kg N ha^{-1} (data not shown). By comparison, added mineral nitrogen had a statistically significant positive effect on certain yield parameters. Yet, this effect was relatively modest in magnitude (Table 4). For instance, there was essentially no yield difference between 70 and 130 kg N ha^{-1}, which could be due in part to the fact that the field was heavily fertilised with nitrogen (which is customary with current French farming practices) in years prior to the start of the experiment. The overall reduction of mineral nitrogen levels in soil observed over the 2001–2002 period (Table 7) is consistent with this hypothesis. Thus, the experiment is relevant from the point of view of switching from heavy to more sustainable N fertilisation levels, and in this context it is likely that the experiment did not last long enough to reach a situation where N was limiting-enough and where the inoculant could have an effect also on yield.

Among key functional groups in maize-based agroecosystems, the focus was put on (i) mycotoxigenic *Fusarium* spp. colonising maize shoots and (ii) soil fauna noticeably faunal groups involved in the turnover of crop residues. Colonisation of maize roots by *A. lipoferum* CRT1 was not expected to influence mycotoxigenic *Fusarium* spp. or soil fauna, at least directly, but arguably both may be indirectly affected, noticeably if inoculation has an impact on plant physiology and/or composition. Nitrogen fertilisers affected shoot N content in 2001 (Table 6), but inoculation did not. Whether inoculation had any effect on shoot composition (other than nitrogen content) is not known but cannot be ruled out. Indeed, *A. lipoferum* CRT1 produces phytohormones and displays 1-aminocyclopropane-1-carboxylate deaminase activity (unpublished results). The latter property enables PGPR strains to interfere with ethylene metabolism in plants (Glick et al., 1998), and it is known that ethylene concentration can modulate plant susceptibility to certain diseases (Abeles et al., 1992). In addition, plant hormonal effects have been involved in the induction by several PGPR strains of plant resistance responses to pathogens (Pieterse et al., 2003), but this has not been documented for *Azospirillum*. Fusarium head blight is caused by different mycotoxigenic *Fusarium* species. These fungi survive and sporulate on crop residues, thereby facilitating contamination of the next crop. In this study, although *F. equiseti* was found in maize residues, only *F. graminearum* was isolated from cobs. *Fusarium* symptoms were not found in stems and were only moderate

on cobs. However, DON contamination of cobs was high, as it was of the same order of magnitude as the tolerance level set for grain commercialisation in certain countries. In Switzerland for instance, this tolerance level (determined following a slightly different protocol) is of $1\,\mu g\,g^{-1}$. Previous work has shown that increasing nitrogen fertilisation in wheat (from 0 to $80\,kg\ N\,ha^{-1}$) enhanced both severity of Fusarium head blight and DON levels (Lemmens et al., 2004), which was not confirmed here on maize. Little is published on mycotoxins in the case of maize (Reid and Sinha, 1998; Reid et al., 2001). Aflatoxin contamination of maize kernels was reduced by the application of nitrogen fertilisers (Tubajika et al., 1999). Taken separately, *Azospirillum* inoculation and nitrogen fertilisation had little or no effect on disease symptoms and DON levels in the current work. However, in the presence of inoculated *A. lipoferum* CRT1, nitrogen fertilisation significantly reduced disease symptoms and DON levels. Further work will be needed to confirm and understand the scientific basis of this phenomenon.

Inoculation had small but significant effects on decomposition (at the third sampling, in plots that did not receive mineral nitrogen) and nitrogen mineralisation (at the second and third samplings, in plots at the intermediate nitrogen fertilisation level) of maize leaves in litter bags (Table 10). These effects were smaller than those of nitrogen fertilisation. The results of the study confirm previous findings that early litter decomposition is greatly influenced by the initial composition of organic residues, especially nitrogen content (Taylor et al., 1989; Smith and Bradford, 2003). Indeed, correlation analysis indicated that a higher initial nitrogen content resulted in accelerated litter decomposition.

Soil fauna plays a critical role in the regulation of microbial decomposition processes by chopping crop residues and feeding on microorganisms (Wardle and Lavelle, 1997), and here inoculation could have had an impact on faunal populations if crop residue quality and/or turnover were affected. This hypothesis is consistent with the finding that inoculation had a (small) effect on colonisation of litter bags by microarthropods (Table 9). Key macrofaunal groups of the soil food web were also investigated. Among them, earthworms are active organic matter recyclers and are important for soil functioning (Lavelle and Spain, 2001). As their biomass was not affected by inoculation with *A. lipoferum* CRT1, they were probably not responsible for the treatment differences observed in the decomposition process, unless earthworms differed in activity level and/or exhibited feeding preferences (Bonkowski et al., 2000). Epigeic macroarthropods include a few phytophagous species and a large number of beneficial species (parasitic, predatory or detritophagous). The main polyphagous predators on cultivated land belong to the *Carabidae*, *Staphylinidae* and *Arachnidae* families; they may occur at high densities and become active quite early in the season, although the latter family tends to develop a bit later. Certain carabid species seem to be effective predators when the aphid populations are expanding in springtime (Chabert et al., 2002). Here, no impact of inoculation with *A. lipoferum* CRT1 was found when monitoring soil macrofauna (Table 8), indicating that the small effects seen on decomposition and microarthropod colonisation of crop residues did not translate at higher ecological levels in the soil food web.

In conclusion, the use of an *Azospirillum* PGPR inoculant (in parallel with the decrease of nitrogen fertilisation levels) enhanced root growth and development but did not influence yield. Ecological effects of inoculation on mycotoxigenic *Fusarium* spp., soil fauna and/or decomposition of crop residues were either small or absent. When an effect of inoculation was seen, it was smaller than that of mineral nitrogen fertilisation (e.g. on soil microarthropods and on decomposition, nitrogen mineralisation and mesofaunal colonisation of maize leaf residues), but interactions between both factors were often significant (e.g. when considering mycotoxigenic *Fusarium* spp. or microarthropods), and this will be important to take into consideration in future studies.

Acknowledgements

This work was supported by the European Union (project EcoSafe), the CNRS (programme 'Impact des biotechnologies dans les agro-écosystèmes'), the Ministère de l'Aménagement du Territoire et de l'Environnement (programme Pnetox), the PAI project 'Germaine de Staël'. Funding for HEZ was provided by the Research Ministry in Egypt. We are indebted to the experimental farm at La Côte St André (Isère, France) for help with the field experiment. We thank M. Mbitikon, A.E. Kara-Micho and E. Rouffanche (Université Lyon 1) and G.A. Hedjaroude (University of Tehran, Iran) for technical assistance, and L. Jocteur Monrozier and S. Nazaret (Université Lyon 1) for useful discussions. This paper is dedicated to the late Nicole Poinsot-Balaguer (CNRS Marseille).

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