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MASTRETTA, Chiara; Taghavi, Safiyh; VAN DER LELIE, Daniel; Mengoni, Alessio; Galardi, Francesca; Gonnelli, Christina; BARAC, Tanja; BOULET, Jana; WEYENS, Nele & VANGRONSVELD, Jaco (2009) Endophytic bacteria from seeds of Nicotiana tabacum can reduce cadmium phytotoxicity. In: INTERNATIONAL JOURNAL OF PHYTOREMEDIATION, 11(3). p. 251-267.

DOI: 10.1080/15226510802432678
Handle: http://hdl.handle.net/1942/9084

ENDOPHYTIC BACTERIA FROM SEEDS OF *NICOTIANA TABACUM* CAN REDUCE CADMIUM PHYTOTOXICITY

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Abstract

Although endophytic bacteria seem to have a close association with their host plant, little is known about the influence of seed endophytic bacteria on initial plant development and on their interaction with plants under conditions of metal toxicity. In order to further elucidate this close relationship, we isolated endophytic bacteria from surface sterilized *Nicotiana tabacum* seeds that were collected from plants cultivated on a Cd and Zn enriched soil. Many of the isolated strains showed Cd tolerance. Sterilely grown tobacco plants were inoculated with either the endogenous microbial consortium, composed of cultivable and non-cultivable strains, single strains or defined consortia of the most representative cultivable strains. Subsequently, the effects of inoculation of endophytic bacteria on plant development, metal- and nutrient uptake were explored under conditions with and without exposure to Cd. In general, seed endophytes were found to have a positive effect on plant growth, as was illustrated by an increase in biomass production under conditions without Cd. In several cases, inoculation with endophytes resulted in improved biomass production under conditions of Cd stress, and in a higher plant Cd concentration and total plant Cd content compared to non-inoculated plants. These results demonstrate the beneficial effects of seed endophytes on metal toxicity and accumulation, and suggest practical applications using inoculated seeds as a vector for plant beneficial bacteria.

Key words: endophytic bacteria, seed, metal toxicity, *Nicotiana tabacum*, Cd, phytoextraction, nutrient balance.

INTRODUCTION

Rhizobacteria are the most studied plant-associated bacteria and are often found to have beneficial effects on plant growth e.g. via the provision of essential elements, inhibition of colonization by pathogenic microorganisms, or by helping the plant to overcome stress responses to environmental insults (Hallmann *et al.*, 1997). Similar beneficial effects have also been described for endophytic bacteria (for review see Mastretta *et al*., 2006). For their practical application, plant growth-promoting bacteria are frequently applied via seed coatings. Interestingly, not much information is available about plant beneficial endophytic bacteria isolated from seeds. Cultivable endophytic bacteria have been isolated from the seeds of coffee (Vega *et al.*, 2005), Norway spruce (Cankar *et al.*, 2005), rice (Tripathi *et al.*, 2006) and rapeseed (Granér *et al.*, 2003). However, not much is known about their ecological function. Some of these bacteria were found to have anti-fungal activity (Mukhopadhyay *et al.*, 1996), but pathogenic bacteria were also found to inhabit the seeds (Schaad *et al.*, 1995 and Grum *et al.*, 1998). For example, infection of carrot seed by *Xanthomonas campestris* pv. *carotae* (Kuan *et al*., 1985) involves the bacterium gaining access to an internal part of the seed, for example the embryo, as was also reported for *X. campestris* pv. *Manihotis* (Elango and Lozano, 1980); *Erwinia stewartii* targets the endosperm (Rand and Cash, 1921), while *X. campestris* pv. *malvacearum* entered the seed coat (Brinkerhoff and Hunter, 1963). Barak *et al.* (2002) infected lettuce plants with *X. campestris* pv. *Vitians,* which causes bacterial leaf spot. They concluded that the pathogen had the capacity to enter and translocate within the vascular system of lettuce plants without inducing visible disease symptoms. Seed produced from diseased lettuce plants were externally contaminated at a level of *about* 2% incidence of *X. campestris* pv *vitians*, but internally the seeds were not infected. In this case it seems that the pathogen was stopped at the seed surface. This could suggest a kind of communication between bacteria and plant host. Bacterial cell to cell or bacteria host communication was hypothesized by Espinoza-Urgel *et al.* (2000) when they restored the seed adhesion capacity of *Pseudomonas putida* KT2440 by mutating the *ddcA* of this strain, which codes for a putative membrane polypeptide. Expression of *ddcA* revealed to be

dependent on cell density, on the addition to a conditioned medium and on seeds exudates, suggesting the existence of a quorum-sensing system in this strain.

Strains belonging to the genera *Pseudomonas* and *Rahnella* were isolated from surface sterilized seeds of Norway spruce (Cankar *et al.,* 2005). Strains belonging to the same genera were also found in surface sterilized seeds of yellow lupine (Barac et al., 2004). Both genera represent well-known plant-associated bacteria with growth-promoting properties and biological control potential. It can be hypothesized that endophytic bacteria residing inside the seeds could serve as vectors for transmission of plant beneficial traits. To validate this hypothesis, we tested if endophytic bacteria found in seeds of tobacco could have a beneficial effect on their host plants, especially when grown in "stressing" environments with heavy metals present. More specifically, we examined the effects of the seed endophytes on (1) plant growth and nutrients uptake, and (2) plant-metal interactions for *Nicotiana tabacum* grown on Cd-contaminated soils. Tobacco was chosen because of its known high metal accumulating capacity and the fact that it is being tested at several phytoextraction field experiments in Switzerland and Belgium.

MATERIALS AND METHODS

Seed Collection

The seeds of *Nicotiana tabacum* cv. Badischer Geudertheimer (BaG) used in this study were collected from plants grown on a metal-enriched sandy loamy soil in Rafz, near Zurich Airport in Switzerland. The seeds sown in Rafz were obtained from the Landesanstalt für Pflanzenbau Rheinstetten, Germany. On the Rafz site a phytoextraction-based remediation experiment is running; the soil was contaminated by the application of domestic and industrial sewage sludge in the 1960s. The total metal contents in the soil in 2004, the year of seed collection, were 505, 0.7 and 362 mg kg^{-1} for Zn, Cd and Pb, respectively.

Cultivation of plants

Seeds of the *Nicotiana tabacum* cv. Badischer Geudertheimer (BaG) were completely sterilized. The seed endophytic bacteria mainly seem to inhabit the endosperm; this allowed a complete sterilization of the seeds without damaging the plant embryo. In the sterilization protocol used, the seeds were submerged for 30 s in 70% ethanol, after which they were rinsed once in sterile Millipore water for 30 s. Subsequently, the seeds were placed for 35 minutes in $HOCl^-$ 42%, after which the seeds were rinsed three times for 10 min in sterile Millipore water and dried using sterile filter paper. The seeds' sterility was checked by incubating some of them for 3 days at 30°C on ten times diluted 869 medium (Mergeay *et al.*, 1985), containing per liter distilled water: 1g tryptone, 0.5g yeast extract, 0.5g NaCl, 0.1g D-glucose, 0.0345g $CaCl_2.2H_2O$ (pH 7). Seeds were considered sterile when no bacterial growth was observed after seed germination.

The seeds were incubated on a Petri dish containing MSM0 medium (Sigma) in the plant growth chamber during 3 days in the dark followed by 2 days at normal growth chamber conditions (constant temperature of 25°C (day) - 17°C (night), relative humidity of 60-65% and a 12 h (day) photoperiod provided by Philips TDL 58WT33 fluorescent tubes (photosynthetic active radiation 160 µmol m^{-2} s^{-1}). Germinated seeds were moved into sterile Magenta (Sigma) containing 100ml of MSM0 medium (pH 5.6) supplemented with 5.5g agar per liter. Depending on the test conditions, 10µM $CdCl_2$ was added to the medium, and/or 56.6µl of the bacterial inocula was plated on the surface of the solidified medium. The Cd exposure concentration was determined based on 2 independent preliminary range-finding experiments. At 10 µM $CdCl_2$, a slight but significant growth inhibition was observed. Cadmium was chosen since it is the metal of major concern (taking into account food chain contamination and risk for human health) present in the Rafz soil. It also was remarkable that a high percentage of the bacteria isolated from the seeds were Cd tolerant, while none of them showed zinc tolerance. One plant per Magenta and five replicas (Magenta) were used for each combination inoculum/Cd exposure.

All the plants were kept during 2 weeks under complete sterility in the growth chamber; after this period the covers were removed and replaced with a sterile tissue that allowed gas exchange. The

plants were left to grow for 2 more weeks before being harvested. One plant for each combination of inoculum and/or Cd-exposure was used to check for the survival of the inoculum in the plant, three plants were used to analyze plant heavy metals uptake while all plants were used to determine biomass production.

Seeds surface sterilization and extraction of endophytic bacteria

The seeds were surface sterilized during 30 min using a solution of 1% active chloride supplemented with 1 droplet Tween 80 per 100ml of solution, and rinsed 3 times with sterile Millipore water for 10 min. To check surface sterility, the seeds were rolled on 10-fold diluted 869 medium and subsequently removed. To test for the presence and also extraction of seed endophytes, the same seeds were squeezed in a sterile mortar after adding a sterile 10mM $MgSO_4$ solution. Samples dilutions were plated on ten times diluted 869 medium and were incubated for 7 days at 30°C. Table 1 describes the main characteristics of the cultivable bacteria isolated from the surface sterilized seeds.

Preparation of bacterial inocula

Individual strains, combinations of the most representative cultivable strains (S_a, S_b, S_c, S_e and S_f) and the endogenous consortium (S_d) consisting of both cultivable and non-cultivable endophytes, were used for inoculation of sterilized *N. tabacum* seeds. The strains were grown at 30°C on a rotary shaker in ten times diluted 869 medium (Mergeay *et al.*, 1985). Cells were harvested after approximately 12 hours at a density of 10^{+8} CFU/ml (OD_{660} of 0.5) by centrifugation, washed twice in 10mM $MgSO_4$ and resuspended at a density of 10^{+9} CFU/ml. Defined consortia were composed by mixing equal volumes of individual cultures.

Cultivable bacteria present in the environment account for only 1% to 5% of the total bacteria (Amann *et al.*, 1995). For this reason also the entire extracted endogenous bacterial consortium, including the uncultivable bacteria, was used for inoculation. This approach was used to get an idea

about the general effect of the bacteria that inhabit the seeds on plant growth in the presence and absence of Cd stress. The solution taken directly from the seeds, referred to as S_d, was obtained by squeezing 35 surface sterilized tobacco seeds in 2 ml of $MgSO_4$, and was immediately used to inoculate sterilized tobacco seeds. Dilutions of all the inocula were plated on ten times diluted 869 medium and allowed to grow for 7 days incubation at 30°C in order to determined the CFU ml^{-1} inoculum.

Bacterial heavy metal resistance

The isolated and purified strains were tested for their heavy metal resistance using 284 medium (Schlegel *et al.*, 1961). This medium contained per liter deionised water, 6.06g Tris-HCl, 4.68g NaCl, 1.49g KCl, 1.07g NH_4Cl, 0.43 Na_2SO_4, 0.2g $MgCl_2 \cdot 6H_2O$, 0.03g $CaCl_2 \cdot 2H_2O$, 40mg Na_2-$HPO_4 \cdot 2H_2O$ 1%, 10ml Fe(III)NH_4 citrate solution (containing 48mg Fe(III)NH_4 citrate per 100ml), 1ml microelements solution, final pH 7. Four different carbon sources (1.3 ml 40% glucose, 2.2ml 30% gluconate, 2.7ml 20% fructose and 3ml 1M succinate) were added per liter. The microelements solution contained per liter distilled water: 1.3ml 25% HCl, 144mg $ZnSO_4 \cdot 7H_2O$, 100mg $MnCl_2 \cdot 4H_2O$, 62mg H_3BO_3, 190mg $CoCl_2 \cdot 6H_2O$, 17mg $CuCl_2 \cdot 2H_2O$, 24mg $NiCl_2 \cdot 6H_2O$ and 36mg $NaMoO_4 \cdot 2H_2O$. After heat sterilization, the medium was supplemented with different concentrations of $CdCl_2$ and/or $ZnSO_4$, both metals that are present in increased concentration at the Rafz site. The Minimum Inhibiting Concentration (MIC) for Zn and Cd were determined and defined as the minimal concentration of the heavy metal inhibiting growth of the bacterial strains. Zn concentrations ranging from 0.25mM to 4mM and Cd concentrations ranging from 0.15mM to 1.5mM were tested.

Bacterial DNA extraction

Genomic DNA extraction was performed as described by Bron and Venema (1972). DNA extraction failed with four strains, reported as Not Determined (n. d.) in table 1; this is not unusual

especially when working with strains isolated from natural environments. DNA precipitation was obtained by adding 1/10 volume of 3M Na Acetate pH 6 and 2.5x 100% ethanol (-20°C). The DNA was subsequently fished out and solubilized in ddH_2O. DNA quality and quantity were determined on 0.8% agarose gel.

Box-PCR genomic DNA profile

BOX-PCR was used to discriminate between the different cultivable isolates. The PCR reaction contained 5µl 10x Taq-Buffer, 4µl 10mM dNTP's, 2µl Box1-primer (5'-CTACGGCAAGGCGACGCTGACG-3'), 0.25µl Taq polymerase (1 unit; Invitrogen) and 5µl template, in a total final volume of 50µl. The thermocycling conditions were: 1 min. at 95°C, 35 cycles of 1 min. at 95°C, 1.5 min. at 50°C, 8 min. at 65°C, and a final incubation step at 65°C for 8 min. The obtained PCR products were separated by means of a 2% agarose gel electrophoresis, after which their distinct patterns were used to discriminate between the different strains.

16S rRNA amplification and sequencing

The 16S rRNA gene was amplified using primers P0 (27f sequence: 5' GAGAGTTTGATCCTGGCTCAG) and P6 (1495r sequence: 5' CTACGGCTACCTTGTTACGA) in a PCR reaction, consisting of: 5µl buffer (10x), 2µl $MgCl_2$ (50mM), 1µl of each primer (10µM each, Invitrogen), 1µl 10mM dNTP's (Invitrogen), 0.4µl Taq polymerase (Invitrogen), prepared as a master mix, with addition of sterile ddH_2O till the final volume of 50µl, prior to DNA addition (1µl). Cycling conditions were: 1.5 min at 95°C, 5 cycles of 30 sec at 94°C, 30 sec at 60°C, 2 min. at 72°C, 5 cycles of 30 sec. at 94°C, 30 sec. at 55°C, 2 min. at 72°C, 25 cycles of 30 sec. at 94°C, 30 sec. at 50°C, 2 min. at 72°C and a final incubation step at 72°C for 10 min. (Picard *et al*., 2000). 5µl of the resulting PCR-DNA product was checked by gel electrophoresis on a 0.8% agarose gel. The remaining PCR products were cleaned by GFX PCR DNA and gel band purification kit (Amersham Biosciences) before sequence, using the Prism Big Dye Terminator sequencing kit

(Applied Biosystems, Foster City, CA) with 100 ng of template DNA. The extended sequences were obtained with universal primers 26F and 1392R. DNA sequences were determined on a 16 Capillary DNA Sequencer (Applied Biosystems, Foster City, CA). Taxonomic classifications were determined according to Wang *et al.* (2007) at the Ribosome Database Project II (http://rdp.cme.msu.edu/index.jsp).

Plant sampling

Plant material was collected, keeping the shoots and the roots of the same plant separated, and vigorously washed with deionised water. The roots were washed with 10mM $Pb(NO_3)_2$ at 4°C for 10 min. in order to remove the adhering metals and rinsed three times with distilled water. Plants fresh weight was determined on an analytical balance (Mettler H54). The dry weight was obtained after drying the shoots and roots during 48 hours at 60°C.

Plant metal analysis

Dry plant materials of shoots and roots were separately homogenized and mineralized by wet ashing with a mixture of concentrated HNO_3 and $HClO_4$ (5:2 v/v). Zn, Cd, Fe contents were determined by Inductively Coupled Plasma Optical Emission Spectroscopy (I.C.P.-O.E.S). The total Cd content of roots and shoots (in micrograms) was calculated by multiplying plant Cd concentration with the dry weight of these organs.

Inoculum recovery

Plants were harvested after 4 weeks of growth; leaf, stem, root and rhizosphere samples were kept separately. Plants were removed under sterile conditions out of the Magenta and put into a sterile Petri dish after removing the excess of growth medium. Five ml 10mM $MgSO_4$ was added to root material, and after vigorous shaking for 1 min, 100µl of this solution and its subsequent dilutions were plated to check the inoculum's survival in strict contact with the root system, considering it as

rhizosphere. The biggest leaf was sterilely cut from the plant and surface sterilized using 0.1% active chloride supplemented with 1 droplet of Tween 80 per 100ml solution during 2 minutes, and rinsed 3 times with sterile Millipore water. The leaf was dried using sterile filter paper. In order to verify the efficiency of the sterilization, a 100µl sample of the third rinsing water was plated on 10 times diluted 869 medium. The same procedure was used for stem and root with a 0.5 % concentration of active chloride and a sterilization time of 1 minute for the stem and 2 minutes for the roots. After sterilization, the different plant parts were macerated into 10mM $MgSO_4$ using a sterile mortar. Samples (100µl) and their dilutions were plated on 10-times diluted 869 medium and checked after 7 days for growth at 30°C.

Statistical analysis

All treatments were performed with a minimum of three replicas. The significance of the differences was analyzed by one-way and factorial ANOVA, followed by the Tukey test or the HSD-Tukey test for post-hoc comparisons between unequal samples, using the Statistica 6 software package (StatSoft, 2003). Using the same statistical program, a Mann-Whitney U test was used in case of non-normal distribution. The data referred to concentration, ratios and metal contents were previously transformed using the arctangent function.

The other program used for the analysis of data was the SigmaPlot 8.0 (SPSS, Chicago, IL).

RESULTS

All bacteria tested successfully colonized the plants. Results, concerning biomass production, plant Cd concentration and content, ratio iron – cadmium and Cd translocation factor, are presented as percentage values in comparison to those obtained for the non-inoculated control plants, which were considered as 100%.

Effects of Cd exposure and endophytic inoculation on plant biomass production

Growth parameters (roots and shoots dry weight) were determined in order to evaluate the effects of both Cd exposure and endophytic inoculation on plant development. The analyses were made on four weeks old plants grown in Magenta pots.

Positive effects on shoot and/or root biomass production were mainly observed after inoculation by the consortia (figure 1). Inoculation with the S_f consortium improved biomass production of shoots and roots of both Cd-exposed and control plants; the S_e consortium had a positive effect on the weight of shoots and roots of Cd-exposed plants and the S_c consortium only had a positive effect on shoots and roots of control plants. Inoculation with consortium S_d increased the root weight in absence of Cd (figure 1), while in presence of Cd no effect on the shoot dry weight was observed. On the other hand, inoculation with consortium S_b decreased biomass of the shoots of the Cd exposed plants.

Inoculation with *Sanguibacter* sp. S_d2 improved the development of both shoots and roots of Cd treated plants, resulting in a statistically significant difference with the control plants, while *Pseudomonas* sp. S_d12 and S_d13 slightly increased shoots fresh weight under Cd stress. Inoculation with the other strains did not result in any significant effect.

Effect of inoculation on Cd uptake

In order to evaluate possible effects of endophytic inoculation on Cd uptake and distribution in *N. tabacum*, metal concentrations and contents were determined in shoots and roots of inoculated and non-inoculated plants exposed and not exposed to Cd.

Knowing that Cd is phytotoxic (Steffens, 1990; Sandalio *et al*., 2001), we intended to find a relation between biomass production and Cd concentration. Some clear differences between inoculated and non-inoculated plants could be observed (figure 2). Inoculation with both consortia S_d and S_a lead to increased Cd concentrations and also total Cd content in the shoots. Interestingly, after inoculating with the Cd sensitive strain *Sanguibacter* sp. S_d2 (table 1), a slightly lower Cd concentration, but a higher total Cd content was found in the shoots, probably as a result of an

increased biomass production (figure 2). On the contrary, inoculation with *Pseudomonas* sp. S_d13 which is more tolerant to Cd than S_d2 (table 1), resulted in a slightly decreased plant Cd concentration (figure 2).

Since we initially focused on the potential effect of endophytic inoculation on the Cd content in the easily harvestable aerial plant parts, analysis of the roots was only performed during the last experiment. In the final phase of the work, we widened the objective to all possible effects that the inocula could have on plant growth and elements uptake, thus root metal content was analyzed as well. In general, after inoculation, a decreased root Cd concentration was found. Roots of plants inoculated with *Sanguibacter sp*. S_d2 showed a lower Cd concentration, while roots of plants inoculated with *Enterobacter sp*. S_d17 had a lower Cd concentration and total Cd content (figure 3). Inoculation with *Pseudomonas* sp. S_d13 and the consortium S_f lowered the plant root Cd concentration (figure 3).

Ratios metal-cadmium

Disturbances in uptake and distribution of macro- and micro-nutrients in plants were also shown to be correlated with Cd toxicity (Sandalio *et al*., 2001). By calculating the ratios between metals, more specifically Fe, with Cd it was intended to determine a preferential accumulation of a specific metal in comparison with non-inoculated plants. This would suggest an effect of inoculation on metal distribution. It is known that the uptake of different nutrients improves the growth of plants on contaminated soils (Belimov *et al.*, 2004, Burd *et al.*, 2000; Verkleij and Schat, 1990). It is therefore relevant not only to determine the plants' metal concentrations, but also check if the ratios of essential elements and Cd change in inoculated plants compared to non-inoculated control plants. *Nicotiana. tabacum* inoculated with *Sanguibacter* sp. S_d2 (figure 4) showed a lower Fe/Cd ratio in the shoots compared to non-inoculated plants. This means that when the Cd concentration in shoots increased, at least partly, this had a negative effect on the Fe concentration. On the contrary, the ratio Fe/Cd is much higher in the roots of all the inoculations tested. This suggests that the plants,

relatively, assimilated more Fe than the toxic Cd, and that this was more pronounced in the inoculated plants than in the sterile control (figure 4).

Translocation Factor (T.F.)

The Translocation Factor (T.F.) is defined as the ratio between the metal concentrations in the shoot and the metal concentration in the roots. Especially endophytic bacteria, but eventually also rhizosphere bacteria, may have effects on the translocation of metals from roots to shoots. In Cd exposed *N. tabacum*, inoculation with *Sanguibacter* sp. S_d2, *Enterobacter* sp. S_d17, *Pseudomonas* sp. S_d12 and the consortium S_f increased Cd translocation, compartmentalizing more of this metal in the shoots than in the roots (figure 5). At the same time, *Sanguibacter* sp. S_d2 decreased Zn translocation, resulting in an increased amount of this metal in the roots (data not shown).

DISCUSSION AND CONCLUSIONS

In this paper different parameters were analyzed that should help to better delineate the role played by endophytic bacteria, which are naturally present inside tobacco seeds, in the complex interaction with their host plant and trace elements present in the environment.

In this study, inoculation of *N. tabacum* with both single endophytic strains and consortia did not have a negative effect on plant biomass production. The only exception was observed after inoculation with consortium S_b, that seemed to increase plant sensitivity to Cd, showing a decreased shoots biomass production under Cd stress. In some cases, a positive effect of endophytic inoculation on plant growth was observed, even when plants were exposed to Cd (figure 1). Inoculation with consortia resulted in more pronounced beneficial effects on biomass production (figure 1) compared to inoculation with single strains, suggesting synergistic effects of the different members of the consortia. Similar plant growth promoting effects by endophytic bacteria were observed with yellow lupine (Barac et al., 2004) and poplar (Taghavi et al., 2005). This suggests

that none of the strains is pathogenic and that some of them (e.g. strain S_d2 and consortia S_a, S_c, S_e and S_f.) could be considered as plant growth promoting (PGP). In addition, an increased plant Cd concentration, compared with a non-inoculated control plants, was observed several times (figure 2) suggesting that these bacteria can somehow reduce Cd toxicity through their interaction with plant growth.

It is known that, under imbalanced nutrient conditions, PGP bacteria are key elements in plant establishment. Their use can support eco-friendly crop production favoring a reduction in the use of agrochemicals (Herrera et al., 1993; Glick, 1995; Requena et al., 1997). The means by which PGP bacteria can improve the nutrient status of their host plant includes (a) biological nitrogen fixation (diazotrophy), (b) increased nutrient availability into the rhizosphere through the solubilization of unavailable minerals, and (c) increased plant biomass production via the synthesis of phytohormones. Encouraging data have been achieved by Egamberdiyeva and Höflich (2004), who demonstrated that selected PGP bacteria isolated from the soil of different crop root zones (cotton, wheat, tomato, melon, and alfalfa) were able to increase the growth and nutrient uptake of cotton and pea in nutrient-poor Calcisol soil, compared with the control plants that performed poorly under the same conditions.

There is plenty of evidence for bacterial production of phytohormones. Auxins and cytokinins were found to be produced by strains of *Pseudomonas*, *Enterobacter*, *Staphylococcus*, *Azotobacter* and *Azospirillum.* These substances, together with gibberellins, may be considered as causal agents for altering plant growth and development (Arshad and Frankenberger, 1991; Bashan and Holguin, 1997; Leifert *et al.,* 1994). In addition, PGP bacteria can overcome growth inhibition caused by heavy metal stress via metabolization of 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene (Glick et al., 1994; 1998; 2004). The strains used in our experiments (table 1) were not yet investigated for their eventual phytohormone and ACC-deaminase production.

The majority of the cultivable bacteria that were isolated from the seeds (table 1) belonged to the pseudomonads. The study of their Box-PCR products (table 1) revealed that in many cases they represented distinct species. Therefore, it is reasonable to expect different results from their inoculations. Some of the inocula studied probably can influence nutrient uptake under nutrient imbalanced conditions. In case of S_c, for example, we observed a more than 2 times increased Zn uptake in Cd treated shoots (data not shown). This increased Zn uptake might explain the decreased Cd toxicity that is evidenced by an increased biomass production (figure 1). A similar effect was observed after inoculation by strain S_d13 that decreased Zn (data not showed) and Cd concentrations in roots (figure 2) but increased at the same time Fe concentration in this part (figure 4). Our data suggest that the root is an important organ for interactions between trace elements. A preferred Fe uptake in this plant part was observed for all the inoculations studied while this was found only in one case for the shoot (figure 4).

Most of the inoculations lead to an increase of the Cd translocation factor (figure 5). The plants seem, due to the inoculations, to store more toxic metal into their aerial parts. This suggests that Cd accumulated in the leaves should be less harmful for the plant. However, subcellular localization and speciation of Cd in the leaves were not investigated. In any case, this increased metal translocation factor is interesting from the point of view of increasing the efficiency of phytoextraction, and therefore some of these inocula should be tested in a field trial.

In conclusion it is evident that, even under conditions of Cd stress, seed endophytic bacteria can have a beneficial effect on both biomass production and trace elements (Ca, Fe, Zn) uptake and distribution by their host plant. The results also indicate that it is recommendable to study and use these bacteria as consortia and not only as individual strains. The bacteria composing the consortium, in fact, prove to have a general positive influence on plant development. Of the ten inocula tested, only S_b had a negative effect on biomass production and this only in case of Cd exposed plants. In general, the inocula either as single strains, defined consortia or whole consortia, seem to lower Cd uptake especially into the root part. In case inoculation resulted in an increased

Cd uptake into the shoots, inoculation seemed to reduce Cd toxicity by increasing the uptake of essential nutrients from the growth medium. Our findings for endophytic bacteria isolated from tobacco seeds support the suggestion by Cankar *et al.* (2005) that the seeds can serve as a vector for beneficial bacteria. Many of the endophytic strains used in this study, like *Sanguibacter* sp. S_d2 and *Pseudomonas* sp. S_d12, as well as the consortia like S_a, S_c, S_d, S_e and S_f, seem promising candidates for phytoextraction field trials. All of them increased biomass production and Cd content in the plants, especially of in the shoot part; good biomass yield and high metal accumulation in harvestable plant parts are essential key factors for efficient phytoextraction ((McGrath and Zhao, 2003, Vassilev et al. 2004).

Some important needs for further study on these seed endophytes are the following:

(1) A more detailed study should be performed on the changes that occur in the endophytic seed community in function of storage time of the seeds, this to understand if and what changes occur in the composition of the seed endophytic community, and how these changes influence plant development.

(2) Another open question is the fate of the seed endophytes during development of the plant. For instance, will these seed endophytes become a major fraction of the newly established endophytic community, or will they be outcompeted by the microbial population present in the soil once the start to germinate on a soil?

(3) Also the composition of the next generation of seed endophytes should be investigated in function of the plant growth regime and the composition of the total endophytic community as part of a population dynamics approach.

(4) The improved plant cadmium tolerance and plant metal translocation suggest a field experiment using these strains as inocula.

AKCNOWLEDGEMENTS

The authors wish to acknowledge the European Commission for their support to PHYTAC project (EU PROJECT: QLRT-2001-00429), and the US Department of Energy for their support to research on the application of endophytic bacteria (Contract number DE-AC02-98CH10886). DvdL and ST are presently also being supported by Laboratory Directed Research and Development funds at the Brookhaven National Laboratory, and by the US Department of Energy, Office of Science, BER, project number KP1102010 under contract DE-AC02-98CH10886. CM was also supported by a PhD grant of the Research Council of Hasselt University. JB is presently supported by a PhD grant from the FWO-Flanders. NW is supported by a PhD grant of the IWT-Flanders.

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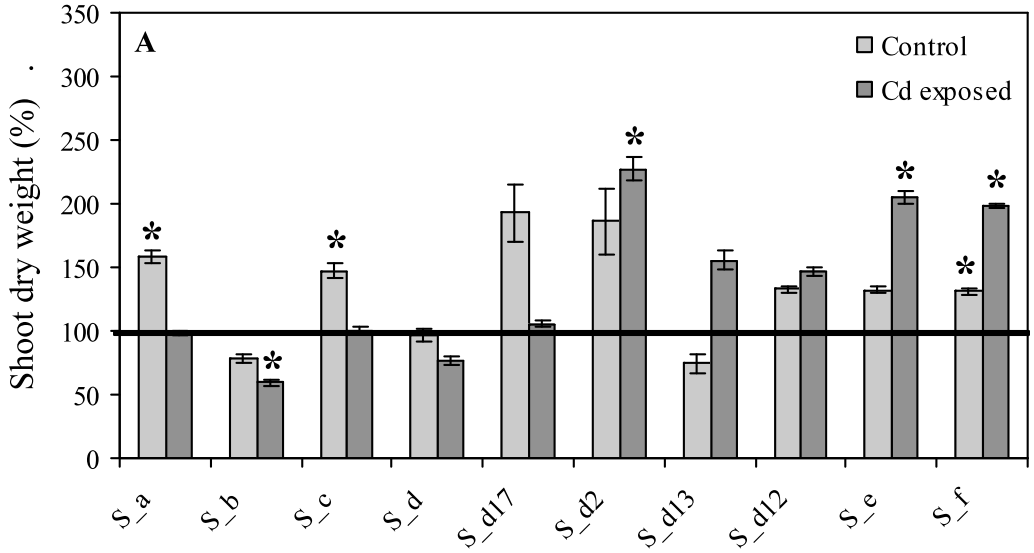
Table 1: Characteristics of bacteria used as inocula. S_d refers to the whole consortium containing both cultivable and uncultivable bacteria. The Minimum Inhibiting Concentrations (M.I.C) values for Cd and Zn (mM) are reported. Similar to column reports the BOX-PCR results resumed, similar letters corresponds to similar PCR fingerprinting which corresponds to equal bacterial species. The last column presents the different inocula concentrations as were used in the inoculation experiments. n. d. = not determined.

Strain n°	Composed by	Sequence result	mM Zn	mM Cd	Box PCR-pattern	Inoculum concentration
S_a	S_a1	*Enterobacter sp.*	<0.25	<0.15	I	$2.2x10^7$
	S_a2	*Enterobacter sp.*	<0.25	0.15	I	
	S_a3	*Xanthomonadaceae*	<0.25	<0.15	B	
	S_a4	nd	<0.25	<0.15		
	S_a5	nd	2	0.6	Q	
S_b	S_b1	*Pseudomonas sp.*	<0.25	0.6	N	$3.2x10^7$
	S_b2	*Pseudomonas sp.*	<0.25	0.6	N	
	S_b3	*Pseudomonas sp.*	<0.25	0.6	N	
	S_b4	*Pseudomonas sp.*	<0.25	0.6	O	
	S_b5	*Pseudomonas sp.*	<0.25	0.6	O	
	S_b6	*Pseudomonas sp.*	<0.25	0.6	O	
	S_b7	*Enterobacter sp.*	<0.25	0.15	R	
	S_b8	*Enterobacter sp.*	<0.25	0.6	R	
	S_b9	*Pseudomonas sp.*	<0.25	0.3	O	
	S_b10	*Pseudomonas sp.*	<0.25	0.45	O	
	S_b11	*Pseudomonas sp.*	<0.25	0.6	F	
	S_b12	*Pseudomonas fulva*	<0.25	0.9		
S_c	S_c1	*Pseudomonas sp.*	<0.25	0.75	N	$6.7x10^7$
	S_c2	*Pseudomonas sp.*	<0.25	0.6	O	
	S_c3	*Pseudomonas sp.*	<0.25	0.75	O	
	S_c4	*Pseudomonas sp.*	<0.25	0.75	O	
S_d entire consortium (cultivable and uncultivable strains)	S_d1	*Pseudomonas sp.*	<0.25	0.6	S	$2x10^2$ (only the cultivables)
	S_d2	*Sanguibacter sp.*	<0.25	<0.15		
	S_d3	*Pseudomonas sp.*	<0.25	0.75		
	S_d4	*Pseudomonas sp.*	<0.25	<0.15	S	
	S_d5	*Stenotrophomonas sp.*	<0.25	0.3		
	S_d6	*Pseudomonas sp.*	<0.25	0.45		
	S_d7	*Pseudomonas sp.*	<0.25	0.3	L	
	S_d8	*Pseudomonas sp.*	<0.25	0.6	L	
	S_d9	*Pseudomonas sp.*	<0.25	0.6		
	S_d10	*Clostridium aminovalericum*	<0.25	0.9		
	S_d11	*Enterobacter sp.*	N.G.	N.G.	I	
	S_d12	*Pseudomonas sp.*	<0.25	1.5		
	S_d13	*Pseudomonas sp.*	<0.25	0.45	G	
	S_d14	*Pseudomonas sp.*	<0.25	0.75	G	
	S_d15	*Pseudomonas sp.*	<0.25	0.3	G	
	S_d16	*Pseudomonas sp.*	0.25	0.9		
	S_d17	*Enterobacter sp.*	0.25	0.45		
	S_d18	nd	0.25	<0.15		
	S_d19	*Pseudomonas sp.*	<0.25	0.3	E	
	S_d20	nd	N.G.	N.G.		
	S_d21	*Pseudomonas sp.*	<0.25	1.05	F	
	S_d22	*Stenotrophomonas sp.*	<0.25	<0.15	D	
S_d17		*Enterobacter sp.*	0.25	0.45		$2.3x10^8$
S_d2		*Sanguibacter sp.*	<0/25	0		$3x10^8$
S_d13		*Pseudomonas sp.*	<0.25	0.45		$5x10^7$
S_d12		*Pseudomonas sp.*	<0.25	1.5		$7x10^8$
S_e	S_d2	*Sanguibacter sp.*				$6.3x10^7$
	S_d17	*Enterobacter sp.*				
	S_d13	*Pseudomonas sp.*				
S_f	S_d2	*Sanguibacter sp.*				$2.7x10^8$
	S_d17	*Enterobacter sp.*				
	S_d12	*Pseudomonas sp.*				
	S_d13	*Pseudomonas sp.*				

Figure 1: Biomass production dry weight of shoot (left part) and root (right part) in plants with different inocula (inocula are specified under each histogram). Histograms with shading refer to the plants grown in presence of 10μM Cd. Means and standard errors are given of three different biological replicas. The data are expressed as percentage of the two respective controls, considering the controls as 100% (horizontal line splitting the graphs). The statistical significance of the results, marked by a star on the histogram, shows a statistical difference from the non inoculated control plants evaluated on the pure data; statistical differences have been calculated in comparison to the respective control values. The statistical significance of the results was confirmed at the 5% level using a two-way and one-way ANOVA model separately exploring inoculums and presence or absence of Cd with non-inoculated plants U Mann-Whitney test was performed when ANOVA analyses was not allowed.


A
Control
Cd exposed
Shoot dry weight (%)
350
300
250
200
150
100
50
0
S_a
S_b
S_c
S_d
S_d17
S_d2
S_d13
S_d12
S_e
S_f

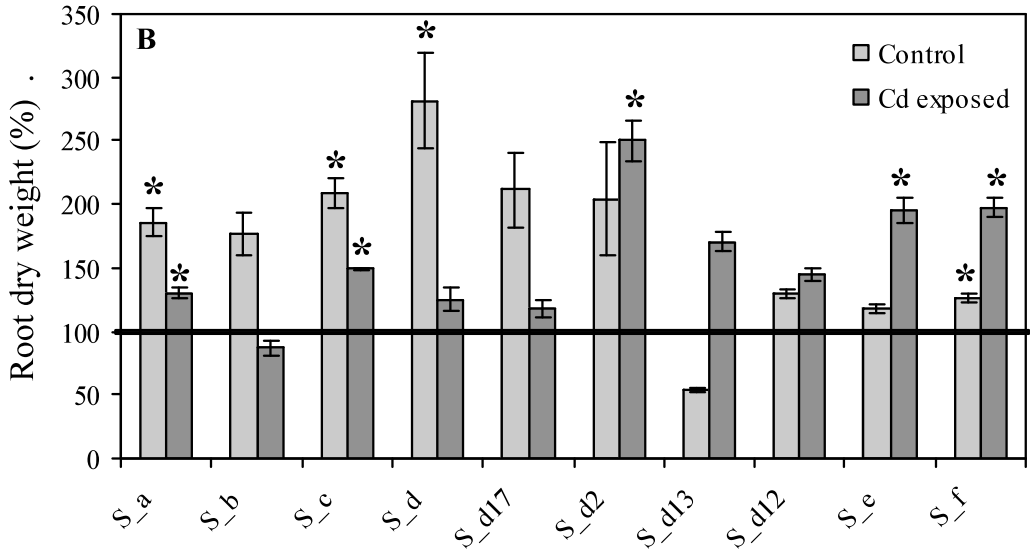
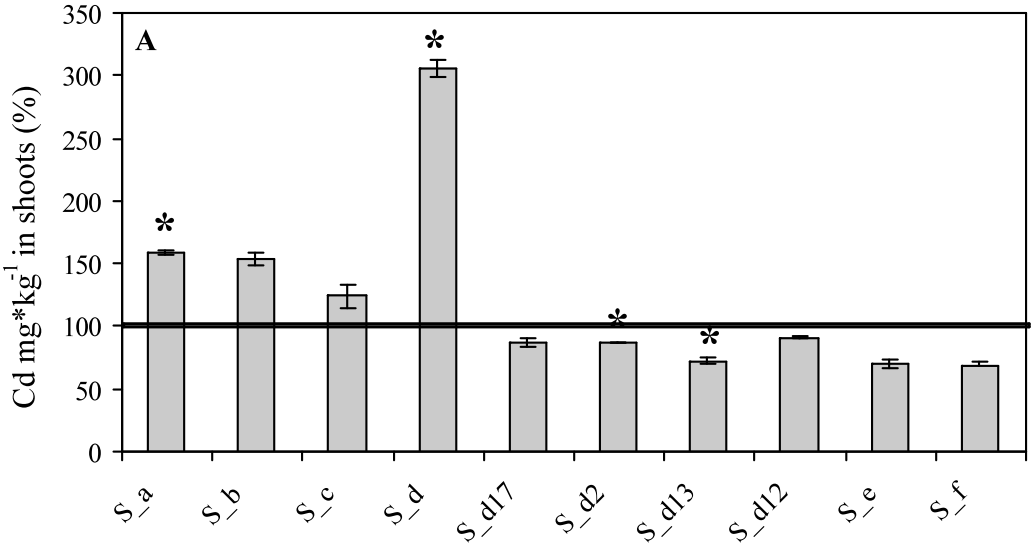

B
Control
Cd exposed
Root dry weight (%)
350
300
250
200
150
100
50
0
S_a
S_b
S_c
S_d
S_d17
S_d2
S_d13
S_d12
S_e
S_f

Figure 2: Cd concentration in shoots (mg kg^{-1} dry weight, left part) and in roots (µg per plant, right part) of plants grown in the presence of 10µM Cd. The different inocula are specified under each bar. Means and standard errors are shown. The data are expressed as percentage of the respective non-inoculated plants (control) considered as 100% (horizontal line splitting the graphs). Indicated by a star on the histogram are the values showing a statistical difference from the non-inoculated control plants evaluated on the pure data confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.


A
Cd mg*kg-1 in shoots (%)
350
300
250
200
150
100
50
0
*
*
*
*
S_a
S_b
S_c
S_d
S_d17
S_d2
S_d13
S_d12
S_e
S_f

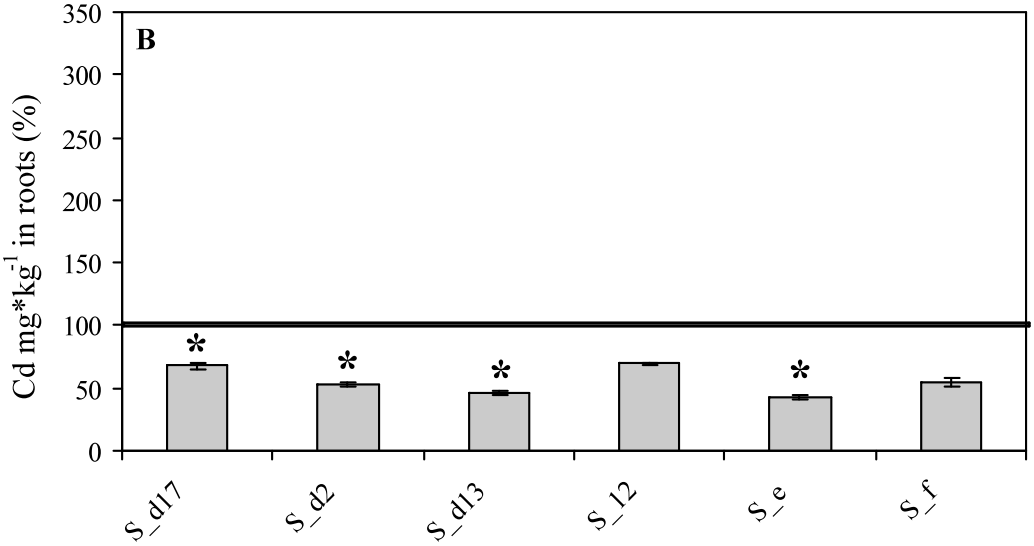
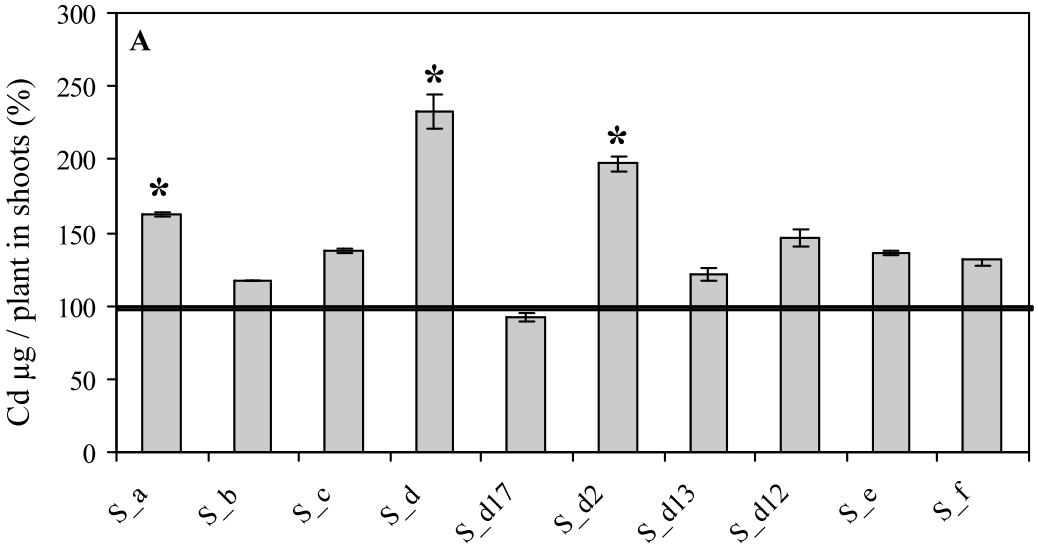

B
Cd mg*kg-1 in roots (%)
350
300
250
200
150
100
50
0
*
*
*
*
S_d17
S_d2
S_d13
S_12
S_e
S_f

Figure 3: Total Cd content in shoots (µg per plant, left part) and roots (µg per plant, right part) of plants grown in the presence of 10µM Cd. The different inocula are specified under each bar. Means and standard errors are shown. The data are expressed as percentage of the respective non-inoculated plants (control) considered as 100% (horizontal line splitting the graphs). Indicated by a star on the histogram are the values showing a statistical difference from the non-inoculated control plants evaluated on the pure data confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.


A
Cd µg / plant in shoots (%)
300
250
200
150
100
50
0
*
*
*
S_a
S_b
S_c
S_d
S_d17
S_d2
S_d13
S_d12
S_e
S_f

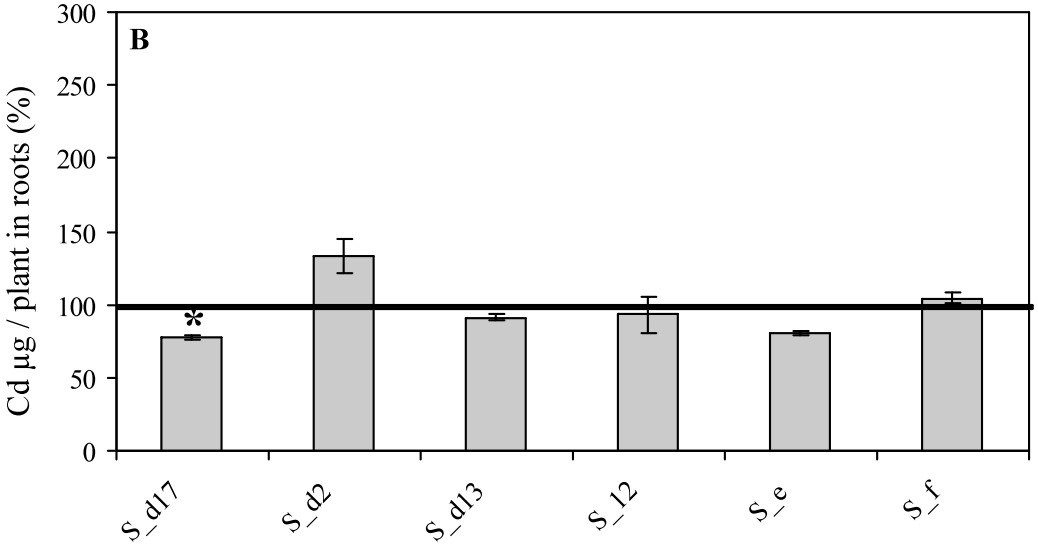
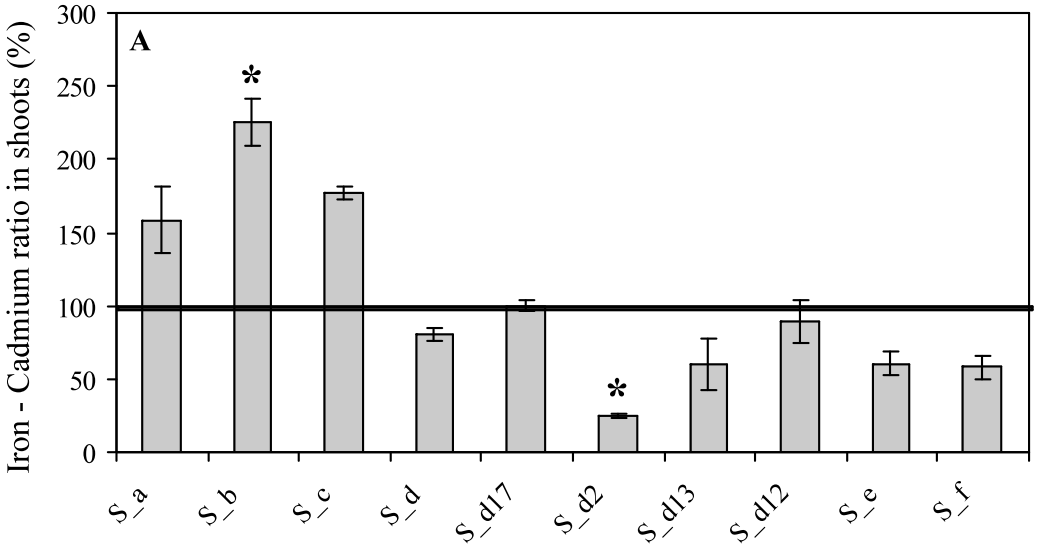

B
Cd µg / plant in roots (%)
300
250
200
150
100
50
0
*
S_d17
S_d2
S_d13
S_12
S_e
S_f

Figure 4: Iron-cadmium ratio in shoots (left part) and roots (right part) of plants grown in the presence of 10µM Cd. The different inocula are specified under each bar. Means and standard errors are shown. The data are here expressed as percentage of the respective non-inoculated plants (control) considered as 100% (horizontal line splitting the graphs). Indicated by a star on the histogram are the values showing a statistical difference from the non inoculated control plants evaluated on the pure data confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.


A
Iron - Cadmium ratio in shoots (%)
300
250
200
150
100
50
0
*
*
S_a
S_b
S_c
S_d
S_d17
S_d2
S_d13
S_d12
S_e
S_f

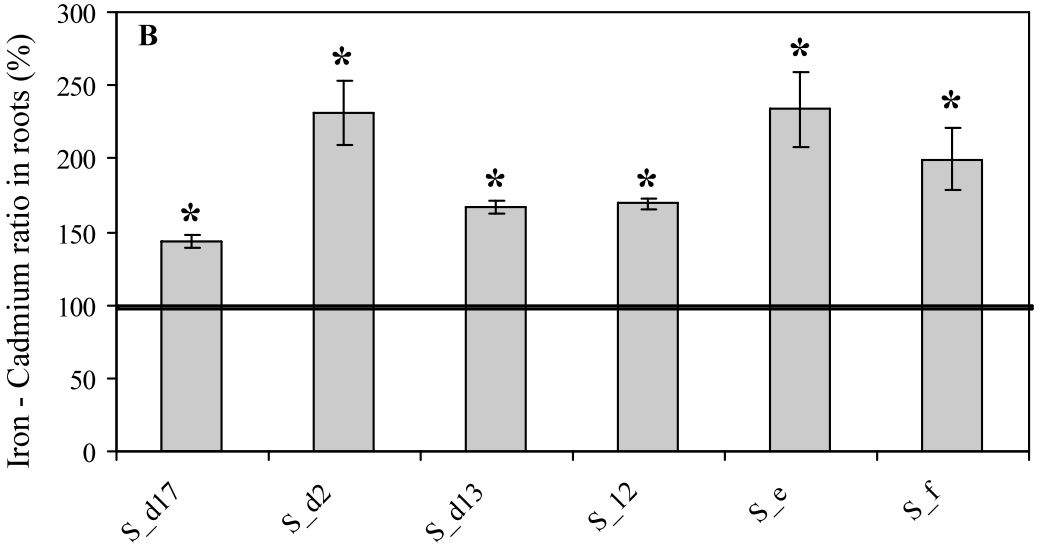
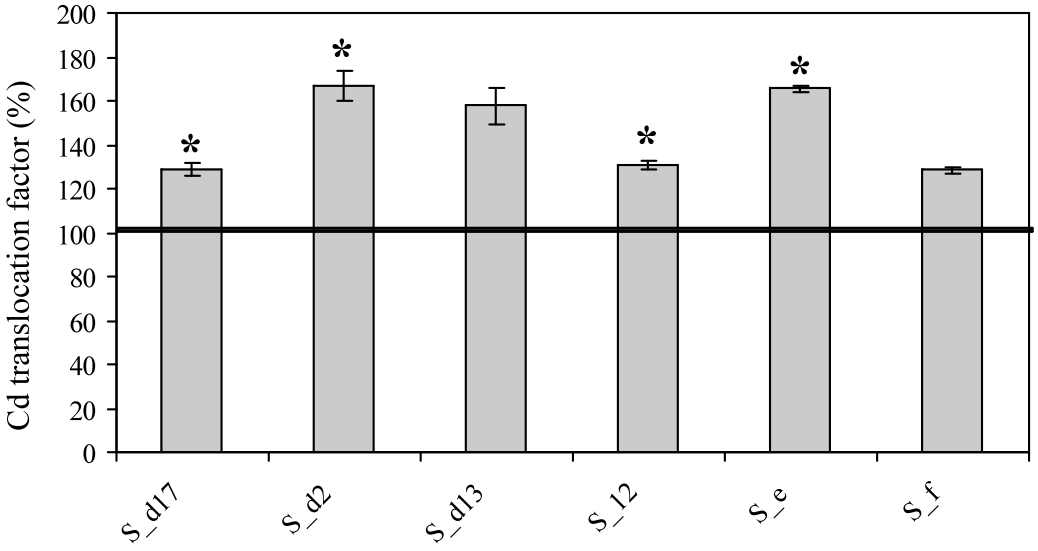

B
Iron - Cadmium ratio in roots (%)
300
250
200
150
100
50
0
*
*
*
*
*
*
S_d17
S_d2
S_d13
S_12
S_e
S_f

Figure 5: Translocation Factor (T.F.) of Cd in plants grown in the presence of 10μM Cd. The different inocula are specified under each bar. Means and standard errors are given of three different biological replicas. The data are expressed as percentage of the respective non-inoculated plants (control) considered as 100% (horizontal line splitting the graphs). Indicated by a star on the histogram are the values showing a statistical difference from the non inoculated control plants evaluated on the pure data confirmed at the 5% level using a one-way ANOVA model separately exploring inocula.


Cd translocation factor (%)
200
180
160
140
120
100
80
60
40
20
0
*
*
*
*
S_d17
S_d2
S_d13
S_l2
S_e
S_f