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Phytochemistry 65 (2004) 1651–1659
PHYTOCHEMISTRY
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Impact of sewage sludges on *Medicago truncatula* symbiotic proteome

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Received 12 January 2004; received in revised form 29 April 2004
Available online 17 June 2004

Abstract

The effects of sewage sludges were investigated on the symbiotic interactions between the model plant *Medicago truncatula* and the arbuscular mycorrhizal fungus *Glomus mosseae* or the rhizobial bacteria *Sinorhizobium meliloti*. By comparison to a control sludge showing positive effects on plant growth and root symbioses, sludges enriched with polycylic aromatic hydrocarbons or heavy metals were deleterious. Symbiosis-related proteins were detected and identified by two-dimensional electrophoresis and matrix-assisted laser desorption ionization mass spectrometry, and image analysis was used to study the effects of sewage sludges on *M. truncatula* symbiotic proteome.

Keywords: Sewage sludges; Proteomics; *Medicago truncatula*; Mycorrhization; Nodulation

1. Introduction

Sewage sludges are highly complex wastes resulting from the treatment of waste waters. These biosolids usually contain high levels of mineral nutrients (mainly nitrogen and phosphorus) and organic matter, leading to improved soil physical properties, a feature which has propelled their use as farm fertilizers (Hall, 1992; Evans, 1998). However, spreading of sewage sludges onto agricultural lands is of great concern, since they may also contain pathogens and parasites (Bertoldi et al., 1982), heavy metals (Beckett and Davis, 1982) and organic chemicals (Berset and Holzer, 1995). In particular, plant–microbe symbiotic interactions involving arbuscular mycorrhizal fungi or rhizobial bacteria can be affected by sewage sludge contaminants. Heavy metals inhibit spore germination, hyphal extension and mycorrhizal development (Hepper, 1979; McGee, 1987; Jacquot-Plumey et al., 2001), and mycorrhizal colonization is decreased in soils polluted with polycyclic aromatic hydrocarbons (PAHs) (Cabello, 1997; Leyval and Binet, 1998). The number of *Rhizobium* in soil and the level of nitrogen fixation in nodulated plants are also decreased by these pollutants (McGrath, 1994; Obbard and Jones, 2001; Wetzel and Werner, 1995). Until now, no studies have been conducted to evaluate the toxicity of sewage sludges on mycorrhizal and rhizobial symbioses at the molecular level. In a previous work, proteomics based on two-dimensional gel electrophoresis and mass spectrometry, allowed to identify some symbiosis-related proteins (SRPs) in the interactions between the model legume *Medicago truncatula* and the arbuscular mycorrhizal fungus *Glomus mosseae* or the nitrogen-fixing bacteria *Sinorhizobium meliloti* (Bestel-Corre et al., 2002). In the present study, more SRPs were detected and identified by refining the technology with a narrower pH gradient and wider second dimension gels. Thorough image analysis was then used to identify the effects of sewage sludges on the levels of these proteins.

2. Results and discussion

2.1. Growth parameters

Shoot and root fresh weights of plants were measured after six weeks of growth (Fig. 1). Due to high variation,

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doi:10.1016/j.phytochem.2004.04.037

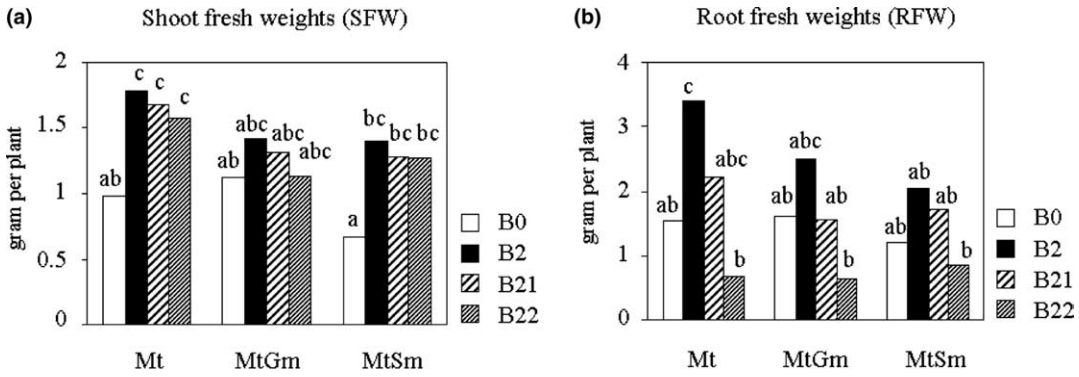

Fig. 1. Shoot and root fresh weights (FW) of *M. truncatula* plants grown in control- or sludge-amended substrate and inoculated or not with *G. mosseae* and *S. meliloti*. Letters indicate statistical groups according to Newman–Keuls tests.

differences between treatments were not always statistically significant, however some tendencies were observed. When plants were grown with the control sludge B2, shoot and root fresh weights were significantly increased by comparison to B0 plants grown without sludge. This effect was previously reported in *M. truncatula* (Jacquot-Plumey, 2001) and directly reflects the nutritious characteristics of sludges. When plants were grown with the PAH-enriched sludge B21 or the metal-enriched sludge B22, fresh weights of roots tended towards a decrease by comparison to B2. These effects were observed whether plants were not inoculated (Mt), or inoculated with *G. mosseae* (MtGm) or with *S. meliloti* (MtSm). Interestingly, Mt plants showed superior biomass to MtGm and MtSm plants, when grown on any of the sewage sludges. These results are contradictory with a previous study in our group (Jacquot-Plumey, 2001), in which no difference was observed between plants grown on the three sludges, and mycorrhizal plants showed improved growth by comparison to the non-inoculated. Such contradictions may be explained on the one hand by the presence in our culture substrate of Epoisses soil, while in the previous study, only sand was used. On the other hand, in our work, plants were fertilized three times per week (versus once) and one tenth-phosphorus was included in the nutrient solution of mycorrhizal plants (versus no phosphorus at all). Therefore in our case, control plants were not suffering any nutritional deficiency, so that no difference could be seen when plants were mycorrhized or nodulated. Meanwhile, the negative effects of contaminated sludges was not masked and the microsymbionts may increase the uptake of chemicals, thereby reducing overall biomass.

2.2. Impact of sewage sludges on mycorrhization

No significant effect was observed on mycorrhization after six weeks of growth (Fig. 2, diagram a). Although a high frequency of mycorrhization (*F*) was found, intensity of colonization (*M*) was rather low. Nonetheless, the colonized parts showed a relatively high amount of arbuscules (*a*), indicating a well-established symbiosis. When the intensity of mycorrhization was related to root fresh weights, differences were in fact detected between the different treatments, with a positive effect of sludge B2, and a negative effect of B21 and B22, by comparison to B2 (Fig. 2, diagram b). In the work of Jacquot-Plumey (2001), a similar tendency was observed for the pollutant enriched sludges by comparison to the control sludge, however control plants without any sludge exhibited a much higher mycorrhization. Again this contradiction may be related to the differences in culture substrates and fertilization since plants cultivated in sand alone and without any phosphorus are more prone to mycorrhization than plants growing with soil and receiving low phosphorus fertilization.

2.3. Impact of sewage sludges on nodulation

When plants were inoculated with *S. meliloti*, no difference in the number of nodules was observed between control plants and those grown with sludges B2 and B21. In response to B22 however, the number of nodules increased significantly (Fig. 3, diagram a). The effects of sludges on nodulation are controversed and were previously shown to depend on the types of soils, sludges, and application levels (Munn et al., 2001). In particular, *S. meliloti* seems preferentially resistant to heavy metals by comparison to other nitrogen-fixing bacteria (Giller et al., 1993). However stimulated nodulation by heavy metal enriched sludge has not been reported previously. In fact, while the total number of nodules per plant was increased in response to the control sludge B2 by comparison to B0, it was decreased in B21 and B22 treated plants by comparison to B2, indicating a negative effect of the pollutants on overall nodulation (Fig. 3, diagram b).

2.4. Identification of symbiosis-related proteins by two-dimensional electrophoresis and mass spectrometry

Root proteins were extracted from plants cultured in the different conditions and separated by two-dimen-

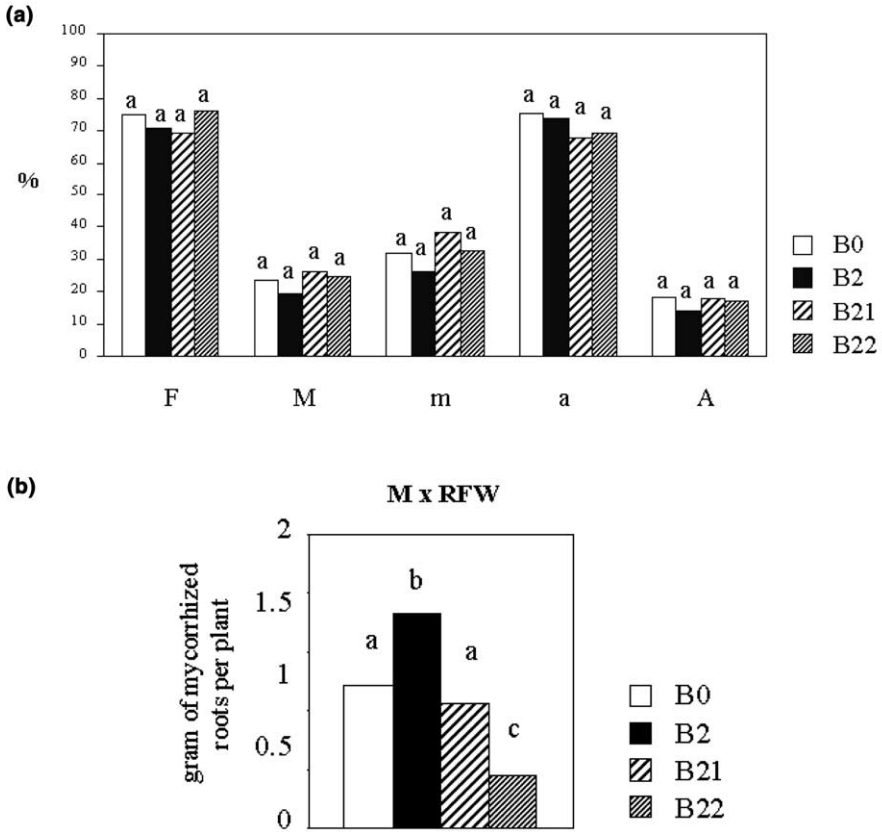

Fig. 2. (a) Mycorrhizal parameters of *M. truncatula* inoculated with *G. mosseae*, after six weeks of growth. *F*: frequency of mycorrhization, *M*: intensity, *m*: relative intensity, *A*: amount of arbuscules, *a*: relative amount of arbuscules. (b) Quantity of colonized roots per plant ($M \times$ RFW). Letters indicate statistical groups according to Newman–Keuls tests.

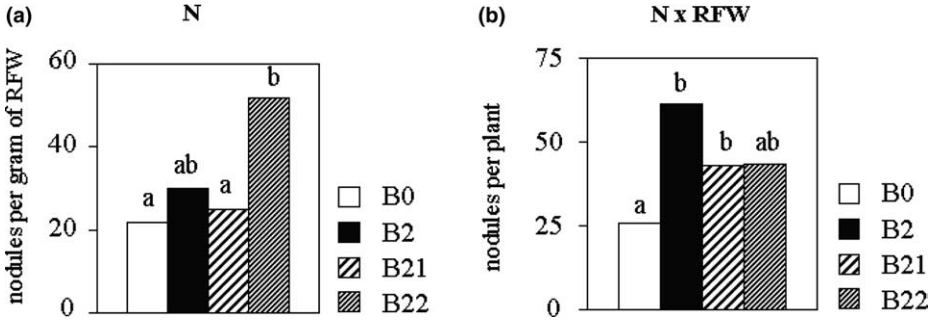

Fig. 3. (a) Number of nodules (*N*) per gram of roots in *M. truncatula* inoculated with *S. meliloti*, after six weeks of growth. (b) Total number of nodules per plant ($N \times$ RFW). Letters indicate statistical groups according to Newman–Keuls tests.

sional electrophoresis. Comparison of the proteome profiles of plants cultured without any sludge led to the detection of several protein spots differentially detected in response to one or the other symbiosis, called symbiosis-related proteins (SRPs). In response to mycorrhization (Fig. 4(a)), 34 SRPs were detected, among which 11 had previously been identified in the same *M. truncatula–G. mosseae* interaction (Bestel-Corre et al., 2002). In response to nodulation (Fig. 4(b)), 41 SRPs were detected, among which 16 had previously been identified (Bestel-Corre et al., 2002). The increase in differentially detected spots can be explained by the use in the present study, of bigger gels combined to a narrower pH gradient, allowing better resolution. Spots corresponding to some of these SRPs were excised from micropreparative gels, digested with trypsin and analyzed by MALDI-TOF mass spectrometry. Peptide mass fingerprint data were used to search databases in order to assign these spots with a function (Table 1).

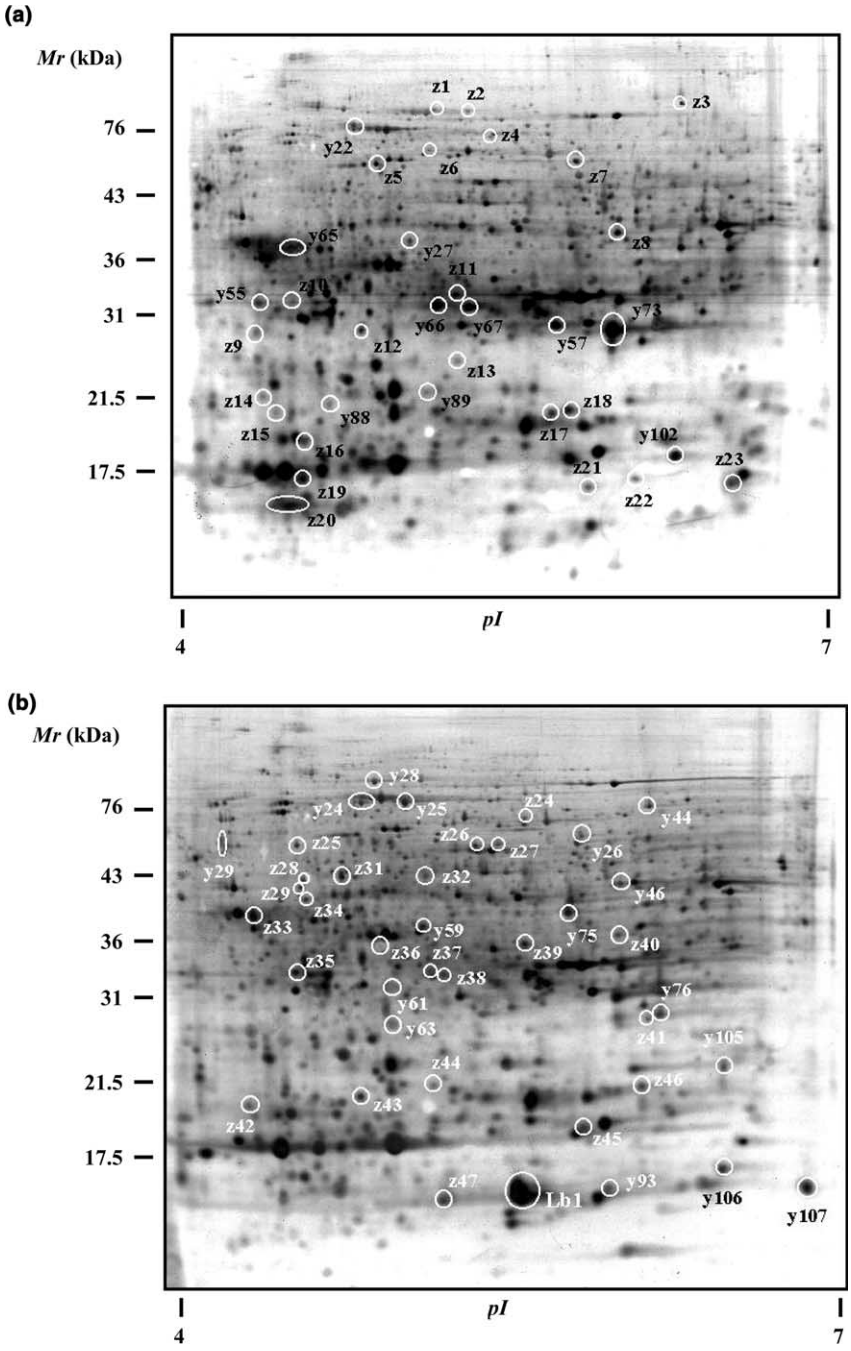

Fig. 4. Two-dimensional electrophoresis profiles of *M. truncatula* inoculated with *G. mosseae* (a) or *S. meliloti* (b), after six weeks of growth. One hundred microgram proteins were loaded and gels were silver-stained. Spot numbers were assigned according to the following rule: y series for spots detected in a previous study (Bestel-Corre et al., 2002) and z were spots detected in the present work.

2.4.1. Mycorrhiza-related proteins

Out of the 23 mycorrhiza-related proteins newly detected, only two were identified in this study. Indeed most of the genomic data available for mycorrhiza are essentially EST sequences, which renders it difficult to obtain high identification scores by peptide mass fingerprinting. This difficulty was previously underlined by Mathesius et al. (2001) and may be overcome by constructing database of EST contigs (Lisacek et al., 2001). Very recently, such a strategy has been proved highly efficient in improving plant protein queries (Valot et al., 2004, in press). However, from our present work, it still seems very difficult to identify mycorrhiza-related proteins with accuracy by peptide mass fingerprinting. This could be related to the lack of such proteins within the *Medicago* database, or to their possible fungal origin. The two plant proteins identified, NAD(p)h dependent 6′-deoxychalcone syn-

Table 1
Identification of symbiosis-related proteins after peptide mass fingerprinting

Spot	Exp. *pI*/*Mr*(kDa)[a]	Hit database	Accession number	Putative function	Score[b]
Mycorrhiza-related proteins					
z8	5.64/39.8	TIGR	TC35803	NAD(p)h dependent 6′-deoxychalcone synthase	90
z12	4.82/27.4	MSDB	Q940F3	Ascorbate peroxidase	78
Nodulation-related proteins					
y28	4.83/65.9	NCBInr	gi|15963935	Heat shock protein 70 (*S. meliloti*)	144
y44	5.63/54.9	NCBInr	gi|16262904	NifK nitrogenase Fe–Mo beta chain (*S. meliloti*)	81
y93	5.45/34.6	TIGR	TC32093	Leghemoglobin	73
z26	5.43/53.9	NCBInr	gi|15966110	GTP binding protein (*S. meliloti*)	163
z27	5.59/53.9	NCBInr	gi|15965092	Elongation factor Tu protein (*S. meliloti*)	162
z31	4.56/44.7	NCBInr	gi|15966233	Leucine specific binding protein precursor (*S. meliloti*)	108
z35	4.45/32.1	NCBInr	gi|15965623	Amino acid binding periplasmic protein (*S. meliloti*)	80
z40	5.64/37.8	NCBInr	gi|15965248	30S ribosomal protein S2 (*S. meliloti*)	125
z43	4.82/17.7	NCBInr	gi|15965326	Transcription elongation factor (*S. meliloti*)	78
z46	5.61/21.2	NCBInr	gi|15964917	Nucleoside-diphosphate-kinase (*S. meliloti*)	101

[a] Experimental *pI*s and *Mr*s were calculated according to those of standard proteins (BioRad 2D standards) co-electrophoresed with the sample.
[b] Score is $-10\,\mathrm{Log}(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 65/72/73 are significant ($p < 0.05$) for databases TIGR/MSDB/NCBInr.

thase and ascorbate peroxidase, are enzymes respectively involved in the phenylpropanoid and detoxification pathways, playing central roles in the defense response of plants to biotic and abiotic stresses. These proteins were previously evidenced during mycorrhization (Spanu and Bonfante-Fasolo, 1988; Harrison

Table 2
Impact of sewage sludges on the amount of symbiosis-related proteins

Spot	Putative function[a]	Spot volumes (%)[b]		
		B2	B21	B22
y57	Glutathione transferase	60 a	22 b	24 b
y65	Cytochrome c oxidase subunit 6b	119 a	43 b	50 b
z12	Ascorbate peroxidase	61 a	22 b	20 b
y22	Peroxidase 1A precursor	61 a	92 b	75 a
y73	Polygalacturonase inhibitor protein	52 a	40 a	59 a
y89	Profucosidase precursor	21 a	20 a	21 a
z8	NAD(p)h dependent 6′-deoxychalcone synthase	119 a	118 a	120 a
y24	Elongation factor Tu	102 a	104 a	148 b
y25	Enolase	142 a	139 a	196 b
y28	Heat shock protein 70	104 a	103 a	144 b
y44	NifK nitrogenase Fe–Mo beta chain	76 a	101 b	124 c
z43	Transcription elongation factor	89 a	81 a	109 b
z46	Nucleoside-diphosphate-kinase	78 a	108 b	129 c
y76	Superoxide dismutase	95 a	111 b	117 b
y93	Leghemoglobin 3	102 a	77 a	134 c
z26	GTP binding protein	100 a	99 a	119 b
z27	Elongation factor Tu	98 a	106 a	122 b
z31	Leucine specific binding protein precursor	103 a	102 a	124 b
z35	Amino acid binding periplasmic protein	91 a	74 a	121 c
Lb1	Leghemoglobin 1	104 a	81 b	112 a
y107	Leghemoglobin 2	121 a	118 a	105 a
y75	Nitrogenase iron protein	122 a	97 b	145 a
z40	30S ribosomal protein S2	102 a	111 a	105 a
y46	Malate dehydrogenase	82 a	99 b	84 a

[a] Identifications were realized in this work or in Bestel-Corre et al. (2002).
[b] Spot volumes in B0 treated plants were normalized to 100% and the volumes of corresponding spots in B2, B21 and B22 treated plants were calculated accordingly. Datas are the means of three replicates. For each spot, numbers with the same letter do not differ significantly.

and Dixon, 1993) as were other defence proteins (Gianinazzi-Pearson et al., 1996; Dumas-Gaudot et al., 2000; Bestel-Corre et al., 2002).

2.4.2. Nodulation-related proteins

While from their identification, mycorrhiza-related proteins were of plant origin, nodulation-related proteins mainly originated from the bacterial symbiont. The only plant protein identified was a third isoform of leghemoglobin. The bacterial proteins comprised another isoform of the elongation factor Tu, a transcription elongation factor, the NifK nitrogenase Fe–Mo beta chain, as well as proteins also detected in *S. meliloti* cultured cells (Bestel-Corre, 2002), a heat shock protein 70, a GTP binding protein, a leucine specific binding protein precursor, an amino acid binding periplasmic protein, the 30S ribosomal protein S2, and a nucleoside-diphosphate kinase. Common proteins were already found between cultured cells of *S. meliloti* and their corresponding bacteroids in *Melilotus alba* nodules (Natera et al., 2000).

2.5. Impact of sewage sludges on mycorrhizal- and rhizobial-related M. truncatula two-dimensional electrophoresis profiles

The identified symbiosis-related protein spots were followed by image analysis comparison between the different treatments. Spot volumes were normalized to 100% in B0 control, and the corresponding spots in B2, B21 and B22 plants were calculated accordingly (Table 2). Two classes of symbiosis-related proteins could be drawn from this experiment. In the first class, proteins for which spots volumes correlated well with the amount of colonization, may be considered as markers of colonization. These corresponded for mycorrhization, to the glutathione transferase, the cytochrome oxidase and the ascorbate peroxidase, and for nodulation, to the elongation factor Tu, enolase, heat shock protein 70, NifK nitrogenase Fe–Mo beta chain, transcription elongation factor, nucleoside-diphosphate-kinase, superoxide dismutase, leghemoglobin 3, GTP binding protein, leucine specific binding protein precursor, and amino acid binding periplasmic protein. Other proteins which were not detected proportionally to the amount of colonization, belonged to the second class of symbiosis-related proteins. For mycorrhization, these comprised the defense related proteins peroxidase, polygalacturonase inhibitor and NAD(P)H dependent 6′-deoxychalcone synthase. Such proteins when expressed at a basal level in mycorrhizal plants, may confer bioprotection towards biotic and abiotic stressors, as it was previously demonstrated for other defense-related proteins with root pathogens (Dassi et al., 1998; Pozo et al., 1999; Slezack et al., 1999). For nodulation, these proteins were the leghemoglobins 1 and 2, the 30S ribosomal protein S2 and the malate dehydrogenase. Differential expression of the various leghemoglobin isoforms has previously been reported which may be related to different roles in nodules (Kawashima et al., 2001). Here, the leghemoglobin 3 may correspond to the isoform induced only in effective nodules.

3. Conclusions

Although pollutant-responsive proteins may have been expected for plants grown in the presence of the pollutant-enriched sludges, in this study, no such additional proteins were detected in response to these sludges. This feature could be related to the relatively low levels of either PAHs or metals used in our experimental design. Nevertheless, following the application of sewage sludges, the expression of symbiosis-related proteins was modulated in mycorrhizal and nodulated plants of *M. truncatula*. Some of these proteins may be used as markers of colonization, since their levels correlated with mycorrhization or nodulation, and they may therefore be used to evaluate the potential toxicity of contaminated soils. Further work will be needed to assess the effects of sludges not only on the levels of colonization but also on the effectiveness of the symbioses. In addition, it will be interesting to carry out studies with model sewage sludge spiked with serial concentrations of single pollutants in order to investigate their level of toxicity.

4. Experimental

4.1. Biological material and growth conditions

Seeds from *M. truncatula* Gaertn. cv Jemalong J5 were surface sterilized and allowed to germinate for three days at 27 °C in the dark. Seedlings were then transplanted into pots containing a sterile mix (2:1, v/v) of quartz sand (sable de Biot, pH 7, particle size 1.6–2.5 mm) and Epoisses soil (clay loam, pH 8.1, 16.6 $g\,C\,kg^{-1}$, 1.8 $g\,N\,kg^{-1}$, 26 mg Olsen $P\,kg^{-1}$), either alone (B0) or in combination with 5% (w/w, fresh weight) sewage sludges (B2, B21 and B22, Table 3). Mycorrhizal inoculation was performed by replacing Epoisses soil with a soil-based inoculum of *G. mosseae* (Nicol. & Gerd.) Gerd. & Trappe (BEG 12) (Dumas-Gaudot et al., 1994). Inoculation with *S. meliloti* strain RCR 2011 was realized by watering the planted seedlings with a 10^4 ml^{-1} suspension of the bacteria. Three pot replicates, each containing three plants, were grown for each treatment. Plants were grown in a growth room under controlled conditions (16 h photoperiod, 23 °C/18 °C day/night temperature, 60% relative humidity, 300 $\mu Einstein\,m^{-2}\,s^{-1}$ photon flux density) and

Table 3
Constituents and characteristics of the sewage sludges used

B2	
DW (%)	18.0
Organic matter (%)	50.1
C_{org} (%)	27.8
N_{tot} (%)	4.0
CaO_{tot} (‰)	96.2
MgO_{tot} (‰)	6.0
P_2O_{5tot} (‰)	96.8
K_2O_{tot} (‰)	4.6
pH_{water}	8.6
PAHs (mg kg^{-1} DW)	
Phenanthrene	1.14
Fluoranthene	0.89
Pyrene	0.82
Benzo(a)pyrene	0.25
Heavy metals (mg kg^{-1} DW)	
Zinc	1436
Copper	594
Lead	352
Nickel	53.3
Cadmium	4.47
B21	
Phenanthrene	396
Fluoranthene	418
Pyrene	70.5
Benzo(a)pyrene	47.9
B22	
Zinc	3643
Copper	939
Lead	909
Nickel	246
Cadmium	26.9

were fertilized three times per week with a modified Long Ashton nutrient solution (Hewitt, 1966) containing one-tenth phosphorus or one-tenth nitrogen for *G. mosseae*- and *S. meliloti*-inoculated plants, respectively, and twice more nitrogen in control and *G. mosseae*-inoculated plants in order to prevent contaminating nodulation. Plants were harvested six weeks after inoculation. To remove roots carefully from the soil mix, pots were placed in tap water. Any remaining soil particles were eliminated by washing roots first with running tap water, and then with deionized water. Randomly collected root parts of plants inoculated with *G. mosseae* were cleared with potassium hydroxyde and stained with trypan blue (Phillips and Hayman, 1970). Mycorrhizal parameters were estimated under light microscopy (Trouvelot et al., 1986). Nodules were counted in plants inoculated with *S. meliloti*. Control, mycorrhizal and nodulated whole root systems were frozen in liquid nitrogen and stored at −80 °C until protein extraction.

4.2. Statistical analyses

Each experiment contained three replicate pots per treatment and was repeated twice. Mean datas were compared with the Newman–Keuls test using SAS software.

4.3. Protein extraction and solubilization

Proteins were extracted according to Bestel-Corre et al. (2002). For each sample, 1 g of *M. truncatula* roots was ground with liquid nitrogen and homogenized in 10 ml of 0.5 M Tris–HCl pH 7.5 lysis buffer containing 0.7 M sucrose, 50 mM EDTA, 0.1 M KCl, 10 mM thiourea, 2 mM PMSF and 2% (v/v) β-mercaptoethanol. Ten millilitre of Tris pH 8 saturated phenols were added. After mixing for 30 min the phenolic phase was separated by centrifugation and rinsed with another 10 ml of lysis buffer. Proteins were precipitated overnight at −20 °C after adding five volumes of methanol containing 0.1 M ammonium acetate. The pellet recovered by centrifugation was rinsed with cold methanol and acetone, dried under nitrogen gas and resuspended in 400 μl of solubilization buffer containing 9 M urea, 4% (w/v) CHAPS, 0.5% (v/v) NP-40, 100 mM DTT and 2% (v/v) IPG Buffer pH 3–10 (Amersham Pharmacia Biotech). Lipids and nucleic acids were removed by ultracentrifuging during 30 min (Beckman Airfuge, 30 psi) and protein content of the supernatants was quantified by the method of Ramagli and Rodriguez (1985) using ovalbumin as a standard. Samples were stored at −80 °C before electrophoresis.

4.4. Two-dimensional gel electrophoresis

Precast 18 cm pH 4–7 IPG strips (Amersham Pharmacia Biotech) were rehydrated overnight with 350 μl of buffer containing 8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 2% (v/v) IPG Buffer pH 4–7 and bromophenol blue. For analytical separations, 100 μg of proteins were loaded at the cathodic end of strips and allowed to focus at 20 °C for 40 kV h using a gradually increasing voltage. Each gel was repeated at least twice for each plant replicate. For micropreparative separations, 500 μg of proteins were used and focusing was extended to 88 kV h. After isoelectric focusing, IPG strips were either stored at −80 °C or immediately equilibrated according to Görg et al. (1987). Strips were then transferred onto vertical slab 12% SDS–polyacrylamide gels and electrophoresis was run at 10 °C for 14 h at 100 V, or until the dye front reached the bottom of the gel.

4.5. Staining procedures and image analysis

Two dimensional analytical gels were silver stained according to the procedure of Blum et al. (1987). Gels were scanned at 300 dpi with a laser image scanner (Amersham Biosciences) and images were saved as 16-bit TIFF files. Image analysis was performed using Image Master 2D Elite software (Amersham Biosciences).

Intensity calibration was performed with Kodak optical diffuse density ruler. Spot detection was performed with the following parameters : sensitivity 9048, operator size 47, noise factor 5, background 1. Background subtraction was realized according to the mode of non-spot with 5 as a factor, and spot volumes were normalized to the total spot volume with a multiplication factor of 100. Micropreparative gels were stained with G-250 colloidal Coomassie blue as described by Neuhoff et al. (1988). After washing gels with water, protein spots were excised and stored at 4 °C in sterile deionized water. Spots were cut into small pieces with a sterile scalpel just before use.

4.6. In gel enzymatic digestion and MALDI-TOF mass spectrometry

Spots were digested and analyzed in the Plateforme de Protéomique Végétale, Montpellier. The protocol of *in gel* digestion is adapted from Jensen et al. (1999). Protein spots from the stained gels were excised and transferred to microcentrifuge tubes. The excised fragments were washed successively with water, 25 mM ammonium bicarbonate, HPLC grade acetonitrile/25 mM ammonium bicarbonate (1:1, v/v) and acetonitrile to remove remaining contaminants and to destain the proteins. Gel fragments were dried under vacuum on a centrifugal evaporator. Digestion was carried out overnight at 37 °C with 10 μl of 0.0125 μg/μl trypsin (sequencing grade, modified, Promega, Charbonieres, France) in 25 mM ammonium bicarbonate (pH 7.8). The resulting tryptic fragments were extracted twice with 100 μl of acetonitrile/water (3:2, v/v) containing 0.1% trifluoroacetic acid in an ultrasonic bath for 15 min. The pooled supernatants were concentrated to a final volume of ≈10 μl in a centrifugal evaporator. The tryptic peptides were desalted and concentrated to a final volume of 3 μl with Zip-TipTM C18 (Millipore, Bedford MA, USA), according to the manufacturer's protocol. Alpha-cyano-4-hydroxycinnamic acid matrix was prepared at half saturation in acetonitrile/water (1:1, v/v) acidified with 0.1% trifluoroacetic acid. 0.8 μl of each sample was mixed with 0.8 μl of the matrix and the mixture was immediately spotted on the MALDI target and allowed to dry and crystallize. The analyses were performed on a BiFlex III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Reflector spectra were obtained over a mass range of 600–3500 Da in the short pulsed ion extraction mode using an accelerating voltage of 19 kV. Spectra from 100 to 200 laser shots were summed to generate a peptide mass fingerprint for each protein digest. At least two peptide ions generated by the autolysis of trypsin were used as internal standards for calibrating the mass spectra. Automatic monoisotopic mass assignment was performed using Bruker's SNAP(TM) procedure.

4.7. Database searching and sequence analysis

M. truncatula EST sequences were downloaded from the TIGR database (www.tigr.org/tdb/tgi/mtgi) and mass data were used to screen the NCBInr, MSDB or *Medicago* databases with Mascot software (www.matrixscience.com) either on a local server or through the internet. Carbamidomethylcysteines and oxidized methionines were chosen as variable modifications, two missed cleavages allowed and 50 ppm mass tolerance.

Acknowledgements

The authors would like to thank Sébastien Lenogue and Josette Bonnefoy for excellent technical help, Ghislaine Recorbet for reading the manuscript, and Alain Hartmann for providing the *S. meliloti* strain. We would also like to thank Nicolas Sommerer and Michel Rossignol for MALDI-TOF analyses. Gwénaëlle Bestel-Corre was granted by the ADEME and the Conseil Régional de Bourgogne.

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