Annals of Microbiology, **59 (4)** 827-832 (2009)

DNA extraction from soil: comparison of different methods using spore-forming bacteria and the *swrAA* gene as indicators

Alma BALESTRAZZI*, Martina BONADEI, Cinzia CALVIO, Alessandro GALIZZI, Daniela CARBONERA

Department of Genetics and Microbiology, University of Pavia, via Ferrata 1, 27100 Pavia, Italy

Received 22 June 2009 / Accepted 28 October 2009

Abstract - Soil microcosms seeded with spores of a tracer organism (*Bacillus subtilis* strain PB5332) were used to test five different DNA extraction protocols hereby indicated as A, B, C, D and E. The representativity of DNA samples obtained from each procedure was evaluated by PCR amplification of the *swrAA* gene, unique to PB5332 strain, followed by Southern hybridization with a gene-specific probe. A significant improvement of DNA extraction from spores was obtained using grinding under liquid N_2 associated with sodium-dodecyl sulphate (SDS)-based lysis in presence of 1% hexadecyltrimethylammonium bromide (CTAB; protocol C). The same procedure was tested on soil samples from two distinct greenhouse trials carried out with genetically modified white poplars (*Populus alba* L.) expressing the *StSy* gene for resveratrol production and the *bar* gene for Basta® tolerance, respectively. The representativity of DNA samples recovered from the greenhouse soil was assessed using three spore-forming bacteria (SFB) as tracer organisms. The tracers (SFB-1, SFB-2 and SFB-3) were previously isolated from the same trials classified as members of the genus *Bacillus*. All the tested DNA samples produced the expected amplification products, indicating the presence at the soil level of the tracers and confirming the reliability of the optimized DNA extraction protocol.

Key words: DNA extraction; CTAB; soil; spore-forming bacteria; *swrAA* gene; tracer.

INTRODUCTION

The fate of recombinant DNA sequences released by genetically modified plants in the soil environment has become the subject for extensive experimental work (Bruinsma *et al*., 2003; Levy-Booth *et al*., 2007). Although relevant progress has been achieved in the use of molecular techniques with environmental samples, working with soil is still difficult due to the presence of contaminants that are co-extracted with DNA. Routine techniques for soil DNA extraction and purification need to be further optimized for a more comprehensive study of the fate of plant recombinant DNA and of the DNA cycle in the soil environment. Contaminants, such as humic acids, polysaccharides or urea, show equal solubility properties compared to DNA and therefore their complete removal is impaired in classical extraction protocols (Lakay *et al*., 2007).

Since less than 1% of the total soil-associated microbial populations can be cultured under laboratory conditions (Malik *et al*., 2008), cultivation-independent methods have been developed, which use the total DNA extracted from soil samples (Tyson and Banfield, 2005). Furthermore, a variable fraction of the soil microorganisms is found in the form of endospores, which are generally considered as the hardiest among cell types, able to easily escape treatments for DNA extraction. To date, optimized methods allowing the recovery of nucleic acids from recalcitrant structures are needed for different purposes, among which the environmental risk assessment of genetically modified (GM) plants.

Kuske *et al.* (1998) used different soils seeded with *Bacillus globigii* endospores to test the efficiency of an hot-detergent lysis treatment followed by bead mill homogeneization. The lowest spore concentration detected corresponded to 14 copies of the *B. globigii* genome. A similar procedure, based on the use of a bead mill homogenizer followed by 1% CTAB extraction, was reported by Williamson *et al*. (1999) who recovered DNA from sediment sample inoculated with dilutions of *Clostridium* spore suspensions. PCR amplification of the expected product was successful in sediment inoculated with 25 or more spores. More recently, concerns about the use of pathogens for bioterrorism has prompted extensive studies on the traceability of vegetative cells and spores in soil environment (Saikaly *et al*., 2007).

In our microcosm-based study, *Bacillus subtilis*, a common inhabitant of soil environments (Nagorska *et al*., 2007), was used as tracer organism. The PB5332 strain (*pheA*$^-$ *swrAA*$^+$ *sfp*$^+$) has been intensively studied since these cells show a typical swarming phenotype (Senesi *et al*., 2004). This trait is depend-

* Corresponding Author. Phone: +390382985435; Fax: +390382528496; E-mail: almbal04@unipv.it

ent on the *swrAA*$^+$ mutation and the spreading of the bacteria is facilitated by the production of surfactin, a biosurfactant that reduces the surface tension. Hyper-flagellated swarmer cells from the PB5332 strain secrete surfactin and create a motile film that allows the bacterial population to colonize large areas. The swarming behaviour positively affects the ability of certain soil bacteria to colonize plants (Daniels *et al*., 2004). The *swrAA* gene product, required for swarming in *B. subtilis*, was found to enhance transcription of flagellar genes (Kearns and Losick, 2005), a trait probably required for the hyperflagellated phenotype displayed by swarmer cells. In a recent work, Osera *et al*. (2009) reported that swrAA activates poly-gammaglutamate synthesis in *B. subtilis*. Since the *swrAA* gene has no homologs in any other sequenced genome, apart from *B. subtilis* and its close relatives *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus amyloliquefaciens*, it represents the ideal target for specific amplifications, circumventing the problem of cross-reaction with other common soil organisms.

In the present work, the attention was focused on a soil, classified as sandy loam and commonly found in the Southern part of Lombardy (Italy), which well supports poplar growth. This soil has been used for the cultivation of transgenic white poplars engineered with the *StSy* gene from *Vitis vinifera* and with the *bar* gene from *Streptomyces hygroscopicus*, respectively, in greenhouse trials (Balestrazzi *et al*., 2007, 2008, 2009a, 2009b; Bonadei *et al*., 2009). In the GM poplars expressing the *StSy* gene encoding stilbene synthase, responsible for resveratrol biosynthesis, the amount of resveratrol-like compounds was in the range 180-210 mg kg $^{-1}$ fresh leaf tissue (Balestrazzi *et al*., 2009b). As for the herbicide-tolereant poplars, the genetically modified poplars were tolerant to intense and repeated glufosinate ammonium treatments, maintaining an intact shoot sprouting and a good height increment (Balestrazzi *et al*., unpublished data).

We present an improved protocol for DNA extraction/purification, suitable for the molecular investigation of the soil microbial populations.

MATERIALS AND METHODS

Microcosm experiments. The soil used in this study was classified as a medium textured loamy sand (77.43% sand, 16.93% silt and 5.62% clay; 24.58% organic matter; 62% Water Holding Capacity) with pH 6.85. Soil samples were collected and processed as previously described (Balestrazzi *et al*., 2008). Two distinct microcosm experiments were designed as follows: 5 g of soil were transferred to 50-mL sterile tubes and mixed with a suspension containing 10^4 and 10^8 cells derived from an exponentially growing culture of the *Bacillus subtilis* PB5332 strain (*pheA1*$^-$*swrAA*$^+$*sfp*$^+$) (Senesi *et al*., 2004). A parallel microcosm was established using 50-mL sterile tubes in which 5 g of soil were mixed with a suspension containing 10^4 and 10^8 spores obtained from the same *B. subtilis* strain. In order to obtain sporulation, cells were seeded in SM medium (Nakano *et al*., 1988) supplemented with 10 µM $MnCl_2$, 1 mM $Ca(NO_3)_2$ and 1 µM $FeSO_4$ and the culture was allowed to sporulate by 48 h-incubation at 37 °C under shaking. Spores were harvested by centrifugation, resuspended in sterile distilled H_2O, treated with Lysozyme (300 µg mL^{-1}, Sigma-Aldrich) and with DNase I (1 mg mL^{-1}, Sigma-Aldrich). Finally, spores were washed with 0.1% Sodium Dodecyl Sulphate (SDS), repeatedly rinsed with sterile distilled H_2O and stored at 4 °C. Spore number and viability was determined by plating progressive dilutions (10^{-6}, 10^{-9}, 10^{-10}) of the spore suspension on NB medium (Difco) and incubating for 30 min at room temperature. For each microcosm, three replicates were analyzed while three independent microcosm-based experiments were carried out.

DNA extraction. Different DNA extraction procedures were tested in the present study using soil microcosms seeded with spores of a tracer organism (*Bacillus subtilis* strain PB5332) (Senesi *et al*., 2004). DNA extraction from soil samples was carried out using the following protocols: A) the method described by Zhou *et al*. (1996) which requires the SDS (2%)-based lysis at high temperature (60 °C) in the presence of 1% hexadecyltrimethylammonium bromide (CTAB); B) the same procedure used in A with an increased CTAB concentration (4%); C) the same procedure used in A but the soil sample was grinded under liquid N_2, before incubation in the lysis buffer; D) microwave-based thermal shock (600-700 W, 1 min) in presence of high concentrations of SDS (3%) and polyvinylpyrrolidone (PVP, 1.2%, Sigma-Aldrich) as described by Orsini and Romano-Spica (2001); E) a combined protocol including grinding under liquid N2 and the microwave-based thermal shock as described for protocol D.

Crude DNAs were subsequently purified using the GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences) according to the supplied protocol. This step was repeated once more and finally the purified DNA was collected and stored at -20 °C. For each protocol, three independent DNA extractions (corresponding to the three independent microcosm-based experiments) were carried out. Each DNA extraction was always performed with three replicated samples. DNA quantitation was carried out by means of spectrophotometric analyses combined with evaluation by agarose gel electrophoresis.

Molecular analyses. PCR and Southern blot hybridization analyses were carried out to amplify and detect the *swrAA* gene, using standard procedures (Sambrook *et al*., 1989). Oligonucleotides YVZD-1 and YVZD-2 (Table 1) were used at the following conditions: 94 °C 30 s, 47 °C 30 s, 72 °C 30 s (5 cycles) and 94 °C 30 s, 55 °C 30 s, 72 °C 30 s (20 cycles) (Calvio *et al*., 2005). For each Spore Forming Bacterium (SFB) isolate, PCR amplification of the 16S rDNA sequence was carried out using the following specific oligonucleotide primers: SFB1-FW and SFB1-RV, SFB2-FW and SFB2-RV, SFB3-FW and SFB3-RV (Table 1) (Bonadei *et al*., submitted for publication). The following conditions were used for SFB-1 and SFB-2: 94 °C for 5 min (1 cycle), 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min (35 cycles). In the case of SFB-3 the annealing temperature was 52 °C. Sequencing of PCR products was performed using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, according to manufacturer's indications and the ABI PRISM® 310 Genetic Analyzer (Perkin Elmer Applied Biosystems).

RESULTS

Detection of the *swrAA* gene in soil microcosms seeded with *Bacillus subtilis* cells and spores

DNAs were extracted from microcosms carrying 10^8 bacterial cells and evaluated by PCR in order to serch for the tracer gene *swrAA* (Fig. 1A). For each PCR reaction, the same template amount (30 ng) was used in the same volume (3 µL). Significant differences were observed in the efficiency of product amplification. The DNA sample obtained with procedure A produced a faint signal, quantified by densitometric analysis

TABLE 1 - Oligonucleotide primer sets usedin PCR reactions

Oligonucleotide	Sequence	Amplicon size (bp)
YVZD-1	5′-TTGAAGAGGGCAAGTATTGTG-3′	*swrAA* coding region (338 bp)
YVZD-2	5′-TTATCTCTCTTGCGTCATCC-3′	
SFB1-FW	5′-TAGGTCAGCGGCGGACG-3′	16S rDNA (664 bp)
SFB1-RV	5′-GCGTTCAGTTACAGACCAG-3′	
SFB2-FW	5′-GATGTTAGCGGCGGACG-3′	16S rDNA (674 bp)
SFB2-RV	5′-GCGTCAGTTACAGACCAG-3′	
SFB3-FW	5′-TGATTTAGCGGCGGACG-3′	16S rDNA (672 bp)
SFB3-RV	5′-GCGTCAGTTACAGGCC-3′	

as 10% of the highest signal (Fig. 1A, lane A). The hybridization signal produced by DNA extracted with protocol B (Fig. 1A, lane B) showed an higher intensity. The best performance was obtained with protocol C which uses the SDS-based lysis with 1% CTAB coupled to grinding under liquid N_2 (Fig. 1A, lane C). The intensity of this hybridization signal was similar to that produced by a PCR assay performed with 0.75 ng of *B. subtilis* DNA template, corresponding to 10^8 bacterial genomes (data not shown). This means an efficiency close to 100%. Low PCR efficiencies were found for the microwave-based protocol D and E (Fig. 1A, lanes D and E). In this case the intensity of hybridization signals corresponded to 20 and 5% of the highest signal, respectively. Densitometric analysis allowed precise quantitation for each hybridization signal and the resulting data were use to calculate the relative amount compared with the highest value observed (Fig. 1A, lane C). The same response was observed using soil microcosms with 10^4 cells. In this case the hybridization signals were lower, as expected, with protocol A, B and C but it was not possible to detect the *swrAA* sequence with protocols D and E (data not shown). Protocol C, the most effective, was selected for further experiments performed on soil microcosms seeded with 10^4 and 10^8 *B. subtilis* spores. In addition, protocol E was also tested, with the idea that recalcitrant microbial structures might require a more robust treatment. The amount of *B. subtilis* DNA in each soil microcosm was calculated to be 428.65 x 10^3 pg (10^8 spores) and 42.86 pg (10^4 spores), respectively. When considering protocol C, which allowed to recover an average amount of total soil DNA corresponding to 17 ± 0.89 µg, it was estimated that 756.44 pg (corresponding to 176.46 x 10^3 copies of the *B. subtilis* genome) was the amount of target DNA contained in a 30 ng-aliquot of the template. This was the case of the microcosm containing 10^8 *B. subtilis* spores. For the microcosms seeded with 10^4 *B. subtilis* spores, the calculated DNA amount found in 30 ng was 0.075 pg (17.4 copies of the *B. subtilis* genome). If protocol C is supposed to result into the highest yield (close to 100%), the *swrAA* gene is expected to produce an hybridization signal similar to that obtained with 756 and 0.075 pg of purified PB5332 DNA, respectively. The hybridization signal obtained from soil microcosms seeded with 10^8 *B. subtilis* spores, using DNA extracted with protocol C are shown in Fig. 1B (lane 1). The comparison between this signals and the hybridization

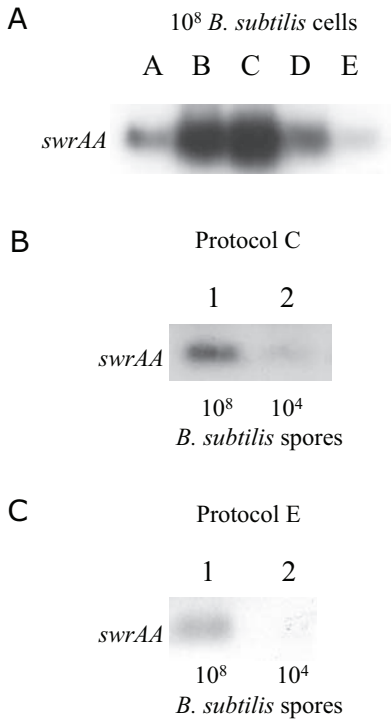

FIG. 1 - Detection of the *swrAA* gene sequence from *Bacillus subtilis* cells and spores in soil microcosms using different DNA extraction procedures.
A: PCR assays were performed using 30 ng of total DNA extracted from soil microcosms spiked with 10^8 *B. subtilis* cells. Southern blot hybridization was carried out on PCR products using the 338-bp *swrAA* fragment as probe. Line A: SDS-based lysis + 1% CTAB at 60 °C (Protocol A); line B: SDS-based lysis + 4% CTAB at 60 °C (Protocol B); line C: grinding + SDS-based lysis + 1% CTAB at 60 °C (Protocol C); line D: microwave-based heat shock + 3% SDS + 1.2% PVP (Protocol D);. line E: grinding + microwave-based heat shock + 3% SDS + 1.2% PVP (Protocol E).
B and C: PCR assays were performed using 30 ng of total DNA extracted from soil microcosms spiked with different amounts (10^8 and 10^4) *B. subtilis* spores.

band produced with the soil microcosms seeded with an equal amount (10^8) of *B. subtilis* cells (Fig. 1A, lane C) clearly demonstrated that the efficiency of DNA extraction from spores was impared, using protocol C. The intensity of the hybridization bands was remarkably reduced (at least 8-fold). This demonstrated that a low lysis efficiency in the case of recalcitrant structures such as the *B. subtilis* spores was associated with the use of protocol C. However the presence of the *swrAA* gene was still detectable in microcosms containing 10^4 spores (Fig. 1B, lane 2). As previously calculated, a single PCR assay carried out with these microcosms contains approximately 17 copies of the *B. subtilis* genome and consequently, this was the resolution threshold of our experimental conditions. Protocol E did not allow a more efficient detection of the target gene, when used with soil microcosms seeded with spores. In this case, the intensity of the hybridization signal obtained with microcosms seeded with 10^8 spores (Fig. 1C, lane 1) was similar to that produced with 10^8 cells (Fig. 1A, lane E) and significantly reduced (approximately 4-fold) compared to the signal obtained with 10^8 spores using protocol C (Fig. 1B, lane 1). This suggests that the combination of different physical treatments (grinding under N_2 plus microwave-based heat shock) was not effective, leading to limited spore disruption. Moreover, when using protocol E, the PCR-Southern hybridization failed to reveal the expected product with microcosms seeded with 10^4 spores (Fig. 1C, lane 2). Control samples used for each microcosm without *B. subtilis* cells, always produced negative results, thus indicating the specificity of the different oligonucleotide primers (data not shown). In the case of procedures A, B and C, the total amount of DNA extracted from each microcosm, quantified by spectrophotometric analysis and evaluation on agarose gel, varied from 17.5 ± 0.89 to 19.3 ± 0.45 µg, while a strong reduction, approximately 10-fold less (from 1.5 ± 0.05 to 1.9 ± 0.09 µg), was observed when the microwave-based approach was used (procedures D and E). A consistent degree of fragmentation was observed in DNA samples extracted by microwave-based protocols (data not shown). Furthermore it is worth noting that the amplification of the 16S rDNA sequence with universal bacterial oligonucleotides was observed in all the DNA samples, indicating that all the templates were accessible to the *Taq* Polymerase, independently of the DNA extraction procedure (data not shown).

The reported data suggest that protocol C might represent an efficient procedure for DNA extraction from soil samples.

Detection of SFB isolates in soil cultivated with transgenic white poplars

Protocol C was then tested with soil samples previously collected from two greenhouse trials established with transgenic white poplars. DNA representativity was assessed using the SFB-1 (DQ988159), SFB-2 (DQ988160) and SFB-3 (DQ988161) isolates as tracer organisms. These environmental isolates, classified as members of the genus *Bacillus* by 16S rDNA-based taxonomy, were recovered from the soil of both greenhouse trials (Bonadei *et al*., submitted for publication).

In order to detect the SFB-1, SFB-2 and SFB-3 isolates, PCR analysis was carried out with specific oligonucleotide primer sets designed from the available 16S rDNA sequences. Molecular analyses were carried out on twelve soil pools obtained from the *StSy* trial and nine soil pools derived from the *bar* trial. All the tested DNA samples produced the expected amplification product, thus indicating the presence of the SFB isolates in the soil (data not shown). Results from PCR analysis performed on the DNA samples extracted from one out of the twelve *StSy* pools and one out of the nine *bar* pools, respectively, are visible in Fig. 2. The reported data demonstrate that protocol C was effective when applied to the loamy sand cultivated with GM poplars and resulted into acceptable representativity of the culturable SFB population inhabiting the same soil.

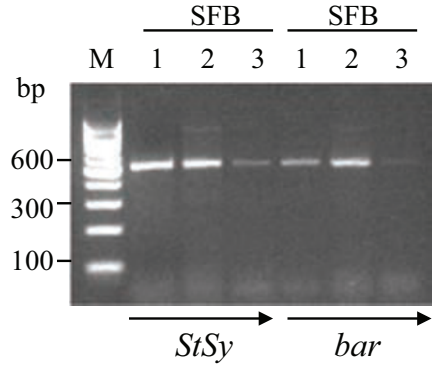

FIG. 2 - Traceability of SFB-1, SFB-2 and SFB-3 isolates in soil collected from the *bar* and *StSy* greenhouse trials. PCR analyses were carried out using the specific oligonucleotide primer sets SFB1-FW/SFB1-RV, SFB2-FW/SFB2-RV and SFB3-FW/SFB3-RV (lanes SFB 1, 2 and 3). M, Gene Ruler™ 100 bp DNA Ladder (M-Medical S.r.l.).

DISCUSSION

Aim of this study was to develop an improved protocol for DNA extraction and purification from a humic acid-rich soil used to establish two extensive greenhouse trials with transgenic white poplars (Balestrazzi *et al*., 2007, 2008, 2009a, 2009b; Bonadei *et al*., 2009).

The efficacy of five protocols in terms of DNA yield and purity was evaluated while attention was focused on the response of recalcitrant microrganisms, particularly the Gram positive bacteria and spores. Stach *et al.* (2001) suggested that a greater DNA quantity recovered does not always equal a greater species richness, demonstrating that there is always the possibility that extracted DNA might derived from the most easily lysed cells. In the present work, the availability of the *B. subtilis* PB5332 strain carrying the unique gene *swrAA* has allowed the investigation on the level of representativity deriving from different lysis procedures.

The level of humic acids in our DNA samples varied before and after purification. Humic substances can compete with nucleic acids for adsorption sites during purification with minicolumns (Rose-Amsageg *et al*., 2001). It is very difficult to remove humic acids and phenolic compounds from DNA samples, and therefore only a combination of purification methods can yield DNA of sufficient purity for successful molecular applications (Rose-Amsageg *et al*., 2001). In our hands, two subsequent purification steps were required to obtain successful PCR amplification which is generally used as an indicator of suitable soil DNA purity (Burgmann *et al*., 2001). Purification was carried out with columns from a commercial kit containing a chaotropic agent that denatures proteins and promotes the binding of double stranded DNA to a glass fiber matrix. It is worth noting that a strong reduction in DNA yields and a significant DNA fragmentation were observed with the microwave-based thermal shock. Orsini and Romano-Spica (2001) reported that the microwave-based

DNA isolation from soil showed the lowest recovery, compared to other environmental samples, however they did not found relevant DNA shearing.

The reported data obtained with soil microcosms confirmed the versatility of the protocol by Zhou *et al.* (1996) as concern representativity, however a significant improvement was obtained by grinding the soil in the presence of liquid N_2 and by increasing the CTAB concentration, which possibly helped to enhance the removal of contaminants, particularly polysaccharides. CTAB might be effective in the case of those bacteria producing extracellular polysaccharides that bind clay particles and form complexes that are maintained in the soil after the death of the microorganisms (Chen, 1998). The increased CTAB concentration (4%) used in protocol B might enhance the formation and precipitation of insoluble complexes with proteins, polysaccharides and cell debris, resulting in a better quality DNA. In our hands, when grinding under N_2 was added to the lysis procedure of Zhou *et al*. (1996), the detection of a limited copy number (approximately 17) of the target sequence *swrAA* released from bacterial spores became possible.

Since the *swrAA* gene has no homologs in any other sequenced genome, apart from *B. subtilis* and its close relatives *Bacillus licheniformis*, *B. pumilus* and *B. amyloliquefaciens*, it represents the ideal target for specific amplifications, circumventing the problem of cross-reaction with other common soil organisms. This was also supported by the fact that no signal was detected in soil microcosms which did not contain the tracer microorganism. Finally, when the improved procedure was tested on the loamy sand collected from the *StSy* and *bar* trials, the SFB isolates previously characterized became the tracer organisms and their presence was confirmed by molecular analyses. Differently from the microcosm-based analyses, it is difficult to make a quantitative evaluation for each SFB isolate found in the soil samples from the greenhouse trials. The reported data suggest that the improved procedure hereby described might become a useful tool in future studies aimed at assessing the environmental impact of GM trees by monitoring novel and specific components of the soil microbial communities.

Acknowledgments

This research was funded by a grant from Regione Lombardia (Divisione Generale Agricoltura). M.B. received a fellowship from Regione Lombardia.

REFERENCES

Balestrazzi A., Bonadei M., Carbonera D. (2007). Nuclease-producing bacteria in soil cultivated with herbicide resistant transgenic white poplars. Ann. Microbiol., 57: 531-536.

Balestrazzi A., Bonadei M., Zelasco S., Quattrini E., Calvio C., Galizzi A., Carbonera D. (2008). Recovery of useful traits from isolates inhabiting an agricultural soil cultivated with herbicide-resistant poplars. Can. J. Microbiol., 54: 201-208.

Balestrazzi A., Bonadei M., Quattrini E., Carbonera D. (2009a). Occurrence of multiple metal-resistance in bacterial isolates associated with transgenic white poplars (*Populus alba* L.). Ann. Microbiol., 59:1-7.

Balestrazzi A., Bonadei M., Calvio C., Mattivi F., Carbonera D. (2009b). Leaf-associated bacteria from transgenic white poplar (*Populus alba* L.) producing resveratrol-like compounds: isolation, molecular characterization and oxidative stress tolerance. Can. J. Microbiol., 55: 829-840.

Bonadei M., Balestrazzi A., Frigerio B., Carbonera, D. (2009). Soil persistence of DNA from transgenic poplar. Environ. Biosafety Res., 8: 79-86.

Bruinsma M., Kowalchuk G.A., vanVeen J.A. (2003). Effects of genetically modified plants on microbial communities and processes in soil. Biol. Fertil. Soils, 37: 329-337.

Burgmann H., Pesaro M., Widmer F., Zeyer J. (2001). A strategy for optimizing quality and quantity of DNA extracted from soil. J. Microbiol. Methods, 45: 7-20.

Calvio C., Celandroni F., Ghelardi E., Amati G., Salvetti S., Ceciliani F., Galizzi A., Senesi S. (2005). Swarming differentiation and swimming motility in *Bacillus subtilis* are controlled by *swrA*, a newly identified dicistronic operon. J. Bacteriol., 187: 5356-5366.

Chen Y. (1998). Electron microscopy of soil structure and soil components. In: Huang P.M., Senesi N., Bollag J-M., Eds, Structure and Surface Reactions of Soil Particles. John Wiley and Sons, pp. 155-182.

Daniels R., VanderLeyden J., Michelis J. (2004) Quorum sensing and swarming migration in bacteria. FEMS Microbiol. Rev., 28: 261-289.

Kearns D.B., Losick R. (2005). Cell population heterogeneity during growth of *Bacillus subtilis*. Genes Dev., 19: 3083-3094.

Kuske C.R., Banton K.L., Adorada D.L., Stark P.C., Hill K.K., Jackson P.J. (1998) Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. Appl. Environ. Microbiol., 64: 2460-2472.

Lakay F.M., Botha A., Prior B.A. (2007) Comparative analysis of environmental DNA extraction and purification methods from different humic acid-reach soils, J. Appl. Microbiol., 102: 265-273.

Levy-Booth D.J., Campbell R.G., Gulden R.H., Hart M.M., Powell J.R., Klironomos J.N., Swanton C.J., Trevos J.T., Dunfield K.E. (2007) Cycling of extracellular DNA in the soil environment. Soil Biol. Biochem., 39: 2977-2991.

Malik S., Beer M., Megharaj M., Naidu R. (2008). The use of molecular techniques to characterize the microbial communities in contaminated soil and water. Environ. Int., 34: 265-276.

Nagorska K, Bikowski M, Obuchowski M. (2007). Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. Acta Biochim. Pol., 54: 495-508.

Nakano M.M., Marahiel M.A., Zuber P. (1988). Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. J. Bacteriol., 70: 5662-5668.

Osera C., Amati G. Calvio C., Galizzi A. (2009). SwrAA activates poly-gamma-glutamate synthesis in addition to swarming in *Bacillus subtilis*. Microbiology, 155: 2282-2287.

Orsini M., Romano-Spica V. (2001). A microwave-based method for nucleic acid isolation from environmental samples. Lett. Appl. Microbiol., 33: 17.

Rose-Amsaleg C.L., Garnier-Sillam E., Harry M. (2001). Extraction and purification of microbial DNA from soil and sediment samples. Appl. Soil Ecol., 18: 47-60.

Saikaly P.E., Barlaz M.A., de los Reyes III F.L. (2007). Development of Quantitative Real-Time PCR assays for detection and quantification of surrogate biological warfare agents in building debris and lechate. Appl. Environ. Microbiol., 73: 6557-6565.

Sambrook J., Fritsch E.F., Maniatis, T. (1989). In: Molecular Cloning: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

Senesi S., Ghelardi E., Celandroni F., Salvetti S., Parisio E., Galizzi A. (2004). Surface-associated flagellum formation and swarming differentiation in *Bacillus subtilis* are controlled by the *ifm* locus. J. Bacteriol., 186: 1158-1164.

Stach J.E.M., Bathe S., Clapp J.P., Burns R.G. (2001) PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. FEMS Microbiol. Ecol., 36: 139-151.

Tyson G.W., Banfield J.F. (2005). Cultivating the uncultivated: a community genomics perspective. Trends Microbiol., 13: 411-415.

Williamson J.L., Rocke T.E., Aiken J.M. (1999). *In situ* detection of the *Clostridium botulinum* type C1 toxin gene in wetland sediments with a nested PCR assay. Appl. Environ. Microbiol., 65: 3240-3243.

Zhou J.Z., Bruns M.A., Tiedje J.M. (1996). DNA recovery from soils of diverse composition. Appl. Environ. Microbiol., 62: 316-322.