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Spore-Forming Bacteria in Soil Cultivated with GM White Poplars: Isolation and Characterization

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ABSTRACT. The impact of transgenic white poplars (*Populus alba* L. cv. 'Villafranca') was assessed on the soil aerobic spore-forming bacteria (SFB). The genetically modified poplars, expressing either the *StSy* gene for resveratrol production or the *bar* gene for herbicide tolerance, were cultivated in greenhouse. The occurrence of SFB was monitored in soil samples collected at eight different timepoints over a two-year period. The total culturable bacterial population of the *StSy* and *bar* trials underwent significant seasonal fluctuations in the range of 10^6–2.5×10^8 CFU/g dry soil and of 10^4–5×10^8 CFU/g dry soil, respectively. Changes occurred also within the culturable SFB population with size varying at 10^3–5×10^4 CFU/g dry soil and 10^2–2×10^5 CFU/g dry soil in the *StSy* and *bar* trials, respectively. No significant differences in the size of the total and SFB culturable populations were observed when comparing each transgenic line with the nontransformed control line while seasonal shifts of soil bacterial populations were evident in both trials. The culturable SFB fraction included three isolates (SFB-1, SFB-2 and SFB-3) classified by 16S rDNA sequence analysis as members of the *Bacillus* genus. According to the reported data, cultivation of both herbicide-resistant and resveratrol-producing GM white poplars did not affect the culturable SFB population at the soil level.

Abbreviations

CFU	colony-forming unit(s)	GM Bt	transgenic maize (corn) genetically modified by incorporation of the *Bacillus thuringiensis* (Bt)-toxin gene
CTRL	control (untransformed) white poplars	PCR	polymerase chain reaction
GM	genetically modified (plants)	SFB	spore-forming bacteria

SFB are versatile microorganisms able to produce spores highly tolerant to adverse environmental conditions, *e.g.*, high temperature and drought (Gorlach-Lira and Coutinho 2007), heavy metal pollution (Smejkalova *et al.* 2003) and intensive fertilizer and pesticide application (Bigelow *et al.* 2002).

In a few cases, dynamics of the SFB population have been monitored in soil cultivated with transgenic plants synthesizing novel products, such as α-amylase or lignin peroxidase (Donegan *et al.* 1999); on the other hand, Ahrenholtz *et al.* (2000) observed the bactericidal effects of transgenic potato plants expressing the T4 lysozyme and harboring root-adsorbed *Bacillus subtilis* cells.

Phytoalexins include resveratrol (3,5,4′-trihydroxystilbene), a secondary metabolite produced by plants in response to pathogen injury and a biomolecule showing relevant health benefits (Pirola and Frojdo 2008). Although several examples of GM plants expressing the *StSy* gene responsible for resveratrol production have been reported (*e.g.*, Delaunois *et al.* 2009), only a few studies related to their environmental impact at the soil level have been described (Bonadei *et al*. 2009; Balestrazzi *et al*. 2009*a*).

The present work focuses on the environmental impact at the soil level of transgenic white poplars (*Populus alba* L. cv. 'Villafranca') engineered with the *StSy* gene for resveratrol production (*StSy* trial) and with the *bar* gene for tolerance to Basta® herbicide (*bar* trial). Weed control in poplar plantations is impaired due to sensitivity toward most common herbicides and this causes reduced productivity during the first years of growth in nurseries and in the field (Meilan *et al.* 2002). The culturable herbicide-resistant bacteria inhabiting the soil from the *bar* trial in the presence and/or absence of herbicide treatment as well as the soil persistence of the recombinant 35SCaMV-*bar* sequence were investigated by Balestrazzi *et al*. (2008, 2009*b*) and Bonadei *et al*. (2009). It is worth noting that the elite clone 'Villafranca' used in this study has been intensively used as model system for several biotechnological applications (Confalonieri *et al.* 2000; Balestrazzi *et al.* 2006) and tested with innovative marker-free gene-transfer technologies (Zelasco *et al.* 2007; Balestrazzi *et al*. 2009*c*).

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MATERIALS AND METHODS

Experimental trials. The *StSy* and *bar* trials were planted in greenhouse in March 2004, with plantlets of *Populus alba* L. (cv. 'Villafranca') produced by *in vitro* micropropagation. The transgenic lines 5EAC1 and 12EAC1 (Giorcelli *et al.* 2004) carry a single copy of the *StSy* gene encoding the stilbene synthase from *Vitis vinifera* (Sparvoli *et al.* 1994) responsible for resveratrol production. The transgenic lines 5P56 and 6EA22P56 carry a single copy of the *bar* gene from *Streptomyces hygroscopicus* encoding phosphinothricin acetyltransferase responsible for herbicide tolerance (Confalonieri *et al.* 2000). All the transgenic lines used harbored the *nptII* marker gene conferring kanamycin resistance. Both the *StSy* and *bar* transgenes and the *nptII* marker gene were under the control of the constitutive 35SCaMV promoter. In each trial, a nontransformed line was tested as control. A schematic representation of the *StSy* and *bar* trials is shown in Fig. 1. The *StSy* trial was established with 180 poplars (60 plants for each line) (Fig. 1A) while the *bar* trial consisted of 54 poplars (18 plants for each line) (Fig. 1B). The single pools (obtained by mixing the soil from six different pots) are indicated by symbols. Twelve and nine soil pools were obtained for the *StSy* and *bar* trials, respectively. The soil temperature was measured at 20 mm depth in the sampling sites, using a Precision Digital Thermometer (*Hanna Instruments*).

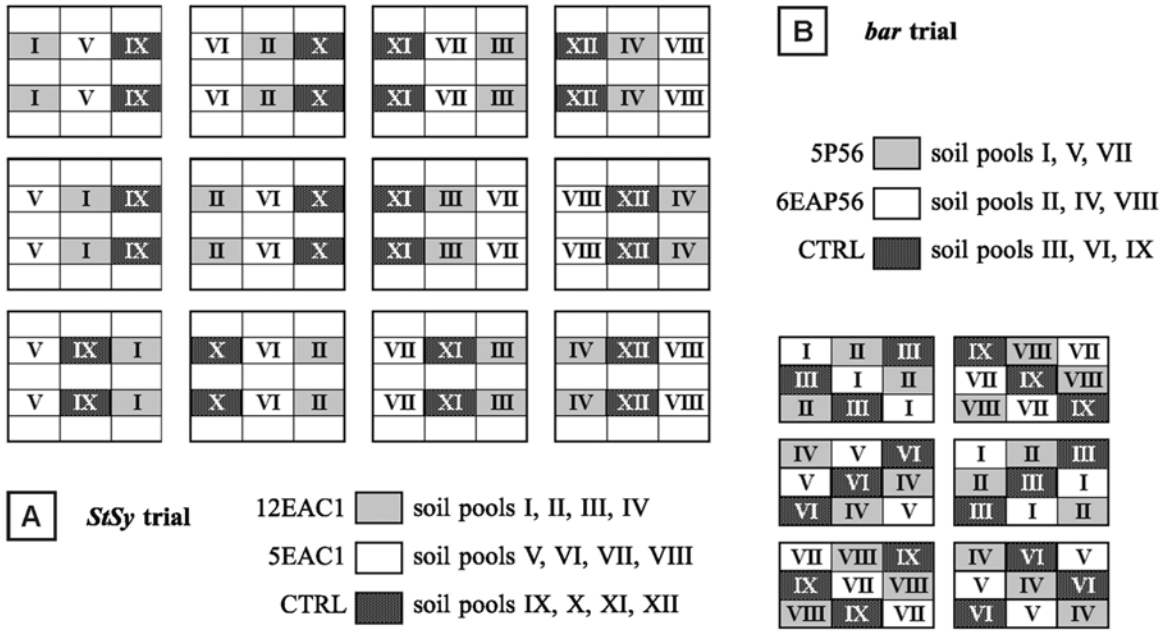

Fig. 1. **A**: Schematic representation of the *StSy* trial which included 180 white poplar plants. For each GM line (5EAC1 and 12EAC1, respectively) and for the nontransformed line (CTRL), 60 plants were utilized. The twelve pools obtained by mixing the soil collected from six different pots according to a randomized scheme are also indicated. **B**: Schematic representation of the *bar* trial which included 54 white poplar plants. For each GM line (5P56 and 6EA22P56, respectively) and for the nontransformed line (CTRL), 18 plants were utilized. The nine pools obtained by mixing the soil collected from six different pots according to a randomized scheme are also indicated.

Soil collection. The soil used was collected from a small noncultivated area surrounded by fields cultivated with fruit trees in the southern part of Lombardy (Italy). The soil (USDA classification: Humic Dystrudept) was mixed with white peat (20 %, Technic; *Free Peat B.V.*, The Netherlands) in order to facilitate rooting. The resulting substrate was classified as medium-textured loamy sand (in %: sand 77.4, silt 16.9, clay 5.6; organic matter 24.5; water-holding capacity 62) with pH 6.85. Soil samples were collected from each pot using a Soil Auger Bucket (*Soilmoisture Equipment Corp.*), transferred to sterile Magenta boxes (77 × 77 × 97 mm; *Sigma Aldrich*) maintained at 4 °C, transported immediately to the laboratory and used <2 h after the removal. Samples were taken to a depth of 180 mm from each pot, diameter of the auger being 30 mm. Samples from six different pots were mixed in order to obtain a total of 12 (*StSy* trial) and 9 (*bar* trial) composite samples (pools).

Microbiological analyses. Soil collection was carried out according to the experimental design (Fig. 1) with the following temporal scheme: T_0 – March 2004, T_1 – May 2004, T_2 – August 2004, T_3 – December 2004, T_4 – March 2005, T_5 – May 2005, T_6 – August 2005, T_7 – December 2005. Soil samples were thus

collected every 3–4 months. Soil samples (1 g) from each pool were resuspended in 10 mL of 0.85 % NaCl and maintained under constant shaking (200 rpm) for 30 min, in order to allow the separation of bacteria from the soil particles. The soil total aerobic culturable bacteria were isolated as follows: Each suspension was serially diluted and 0.1 mL of the 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10} dilutions were plated onto triplicate plates containing Plant Count Agar (PCA; *Oxoid*) supplemented with cycloheximide (100 mg/mL, *Duchefa Biochemicals*). Aerobic SFB were plated on the same medium using dilutions of soil (0.1 mL of the 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-10}, respectively) heated for 10 min at 80 °C. Plate counts were expressed as CFU/g dry soil. Plates were incubated at room temperature and at 37 °C. Plates were maintained for 1 and 2 d, respectively, in order to isolate both copiotrophic and oligotrophic bacteria.

16S rDNA analyses. For taxonomic evaluation PCR amplification of 16S rDNA sequences was done using the universal bacterial primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1522R (5′-AAG GAG GTG ATC CAN CCR CA-3′) (Suzuki and Giovannoni 1996) at the following conditions: 94 °C for 5 min (1 cycle), 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min (35 cycles). All PCR reactions were carried out in a final volume of 30 μL containing 0.2 mmol/L premixed deoxynucleoside triphosphates (*M-Medical S.r.l.*), 1.5 mmol/L $MgCl_2$ and 2.5 U *Taq* DNA Polymerase (DyNAzyme II; *Finnzymes*, *Celbio*), using a T Gradient apparatus (*Biometra*). PCR products were separated on 1.0 % (*W*/*V*) agarose gels (*Duchefa Biochemicals*) and purified using the GFX™ PCR DNA and Gel Band Purification kit (*Amersham Biosciences*). Sequencing of PCR products was performed using an ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit, according to manufacturer's instructions and the ABI PRISM® 310 Genetic Analyzer (*Perkin Elmer Applied Biosystems*).

Statistical analysis was carried out using the Microsoft® Excel2000 (9.0.2812) statistical package for calculating mean and standard error. The results were subjected to analysis of variance (ANOVA) and the means compared by the Duncan's multiple range test (MSTAT-C; *Crop and Soil Science Department*, *Michigan State University*, USA).

RESULTS

The soil temperature (mean values) ranged from 5 to 26 °C in 2004 and 2005, depending on the season (Fig. 2A). Results from microbiological analyses carried out on the *StSy* trial are reported in Fig. 2B. At each time point, the mean values of counts representative of the total culturable bacteria and the mean values corresponding to the SFB population in soil pools from GM lines 5EAC1 and 12EAC1, and from the CTRL line are reported. Soil pools I, II, III and IV (Fig. 1A) were representative of those pots containing the GM 12EAC1 plants. Soil pools V, VI, VII and VIII (Fig. 1A) were derived from pots containing the 5EAC1 GM plants. Finally, soil pools IX, X, XI and XII (Fig. 1A) were obtained from pots cultivated with CTRL white poplars.

Before GM plant cultivation (Fig. 2B; T_0, March 2004) the size of the total culturable population (in the range of 2–3 × 10^8 CFU/g dry soil) did not significantly vary in the tested soil pools ($F = 1.253$, $p = 0.298$). At that time, the SFB population (5–8 × 10^3 CFU/g dry soil) corresponded to ≈40 % of the total culturable bacteria. Following white poplar cultivation, significant changes ($F = 4.217$, $p = 0.003$) in the size of the soil total microbial population were observed. The mean values varied from more than 10^8 CFU/g dry soil (Fig. 2B, T_1, T_5 and T_6; May 2004, May and August 2005) to less than 10^5 CFU/g dry soil (Fig. 2B, T_3; December 2004).

In soil cultivated with white poplars, the SFB populations associated with both the GM and CTRL lines showed size fluctuations. Thus at T_1 (May 2004), the SFB population isolated from pots with CTRL plants was 10^3 CFU/g dry soil while slightly higher values (3 × 10^3 and 2.5 × 10^3 CFU/g dry soil) were found for the 5EAC1 and 12EAC1 lines. The SFB counts at T_2, T_3, T_4 and T_7 (August and December 2004; March and December 2005) were within the reported range while an enhancement was recorded at T_5 (May 2005, 8 × 10^3 CFU /g dry soil) and T_6 (August 2005, 1–2.5 × 10^4 CFU /g dry soil). Considering the changes in the size of the total microbial population, the percentage of SFB calculated for each time significantly varied ($F = 3.952$, $p = 0.003$) in the *StSy* trial. At T_1 (May 2004), the SFB fraction corresponded to ≈30.0 % of the total culturable bacteria. At T_2 (August 2004), the percentage of SFB increased ≤50.0 % but the highest value (71.2 %) was observed at the subsequent time point (T_3, December 2004). During the year 2005 fluctuations in the size of the SFB population, calculated as percentage of the total culturable bacteria, were less consistent, ranging from 41.6 % (Fig. 2B, T_5 and T_6) to 50.0 % (Fig. 2B, T_4 and T_7).

For each sampling date, soil samples in which no plants were cultivated were also analyzed (*data not shown*). The size of the total and SFB populations were lower, comparing with those obtained from cultivated soil but the same seasonal fluctuations were recorded. The above data suggest that cultivation of *StSy*

GM white poplars does not cause significant effects on the size of the SFB population. The only visible changes, occurring simultaneously in soil samples derived from both the GM and nontransformed plants, were related to season.

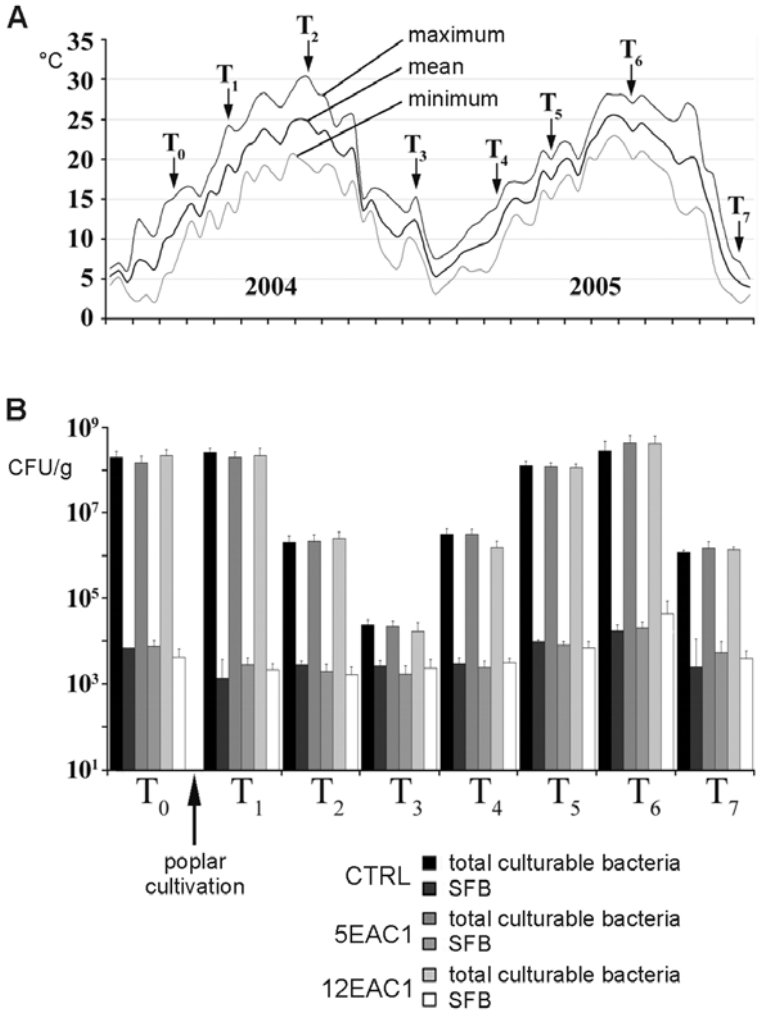

Fig. 2. *StSy* trial. **A**: Temperature (°C) of soil measured at 20 mm depth in the sampling sites at the same day period (noon). Minimum, mean and maximum values are shown. **B**: Amount of the total culturable bacteria and amount of the aerobic SFB fraction inhabiting the loamy sand cultivated with the GM lines 5EAC1 and 12EAC1 and with the nontransformed CTRL line. For each line, the mean values of four soil pools and the standard deviation are shown. T_0 – March 2004; T_1 – May 2004; T_2 – August 2004; T_3 – December 2004; T_4 – March 2005; T_5 – May 2005; T_6 – August 2005; T_7 – December 2005; CFU/g – colony-forming units per g dry soil.

The soil temperature was measured also for the *bar* trial (Fig. 3A). The soil temperature (mean values) ranged from 4 to 27 °C depending on season. The mean values of counts representative of the soil total culturable bacteria and the mean values corresponding to the SFB population in soil pools from the GM lines 5P56 and 6EA22P56 and from the nontransformed (CTRL) line are reported at each time period. Soil pools I, V and VII were derived from pots containing the 5P56 GM plants; soil pools II, IV and VIII were representative of those pots containing the GM 6EA22P56 plants; finally, soil pools III, VI and IX were obtained from pots cultivated with CTRL white poplars (Fig. 1B).

Results from microbiological analyses carried out on soil samples collected from the *bar* trial are shown in Fig. 3B. Before GM plant cultivation (T_0, March 2004), the total culturable population was estimated to be $<10^6$ CFU/g dry soil. At that time the SFB population (2×10^2 CFU/g dry soil) corresponded to ≈25.0 % of the total microbial population. No significant differences ($F = 1.271$, $p = 0.302$) were observed in the tested soil pools. Following GM poplar cultivation, significant fluctuations ($F = 3.968$, $p = 0.003$) were recorded for the size of the total culturable population of the *bar* trial, in relation to season. The size measured at T_1 and T_2 (May and August 2004) ($1–3 \times 10^4$ CFU/g dry soil) progressively increased at T_3 (De-

cember 2004; 8×10^4 CFU/g dry soil), T_5 and T_7 (May and December 2005; 5×10^7 CFU/g dry soil). The highest value was recorded at T_6 (August 2005; 5×10^9 CFU/g dry soil). As for the SFB population, the counts were slightly reduced (in the range of $0.8–1 \times 10^2$ CFU/g dry soil) at T_1 and T_2 (May and August 2004) while an increase (up to 5×10^3 CFU/g dry soil) was observed at T_3 and T_4 (December 2004 and March 2005). The size of the SFB fraction further increased at T_5 (May 2005; 5×10^4 CFU/g dry soil) and then at T_6 (August 2005; 2×10^5 CFU/g dry soil). A drop in the size of the culturable SFB population (10^2 CFU/g dry soil) occurred in winter 2005 (Fig. 3B, T_7). Considering the reported changes in the size

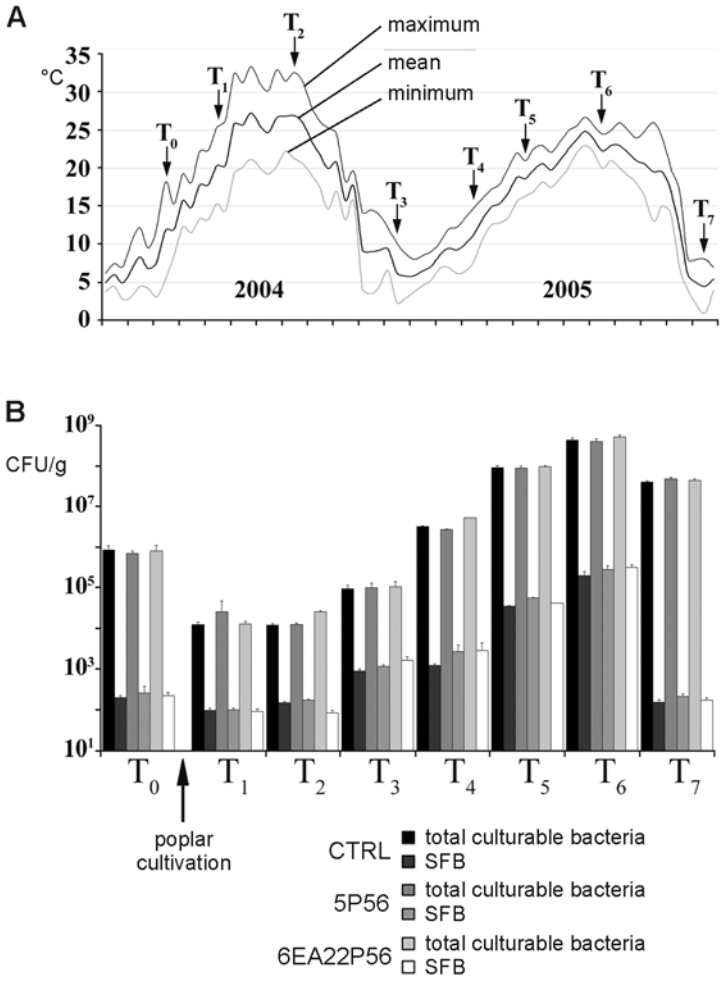

Fig. 3. *bar* trial. **A**: Temperature (°C) of soil measured at 20 mm depth in the sampling sites at the same day period (noon). Minimum, mean and maximum values are shown. **B**: Amount of the total culturable bacteria and amount of the aerobic SFB fraction inhabiting the loamy sand cultivated with the GM lines 5P56 and 6EA22P56 and with the nontransformed CTRL line. T_0 – March 2004; T_1 – May 2004; T_2 – August 2004; T_3 – December 2004; T_4 – March 2005; T_5 – May 2005; T_6 – August 2005; T_7 – December 2005; CFU/g – colony-forming units per g dry soil.

of the total microbial population, the percentage of SFB calculated for each time significantly varied (F = 3.752, p = 0.003) in the *bar* trial. At T_1 and T_2 (May and August 2004), the SFB fraction corresponded to 24.6 % of the total culturable bacteria. The percentage changed then from 50 % (T_3, T_5) and 60 % (T_6) while it was markedly reduced (18.4 %) at T_7 (December 2005). As reported *above* for the *StSy* trial, for each harvesting date, soil samples, in which no plants were cultivated, were also analyzed (*data not shown*). The size of the total and SFB populations was slightly reduced, compared with those obtained from cultivated soil and underwent seasonal shifts. The data suggest that the cultivation of *bar* GM white poplars does not result in significant effects on the size of the SFB population since the only visible changes were related to season.

SFB colonies isolated at each tested time period were picked and recultured bacteria were identified by the sequence analysis of 16S rDNA (50 colonies were analyzed for each soil sample collected at the indi-

cated time points; Figs 2 and 3). The culturable SFB population was represented by three isolates classified as members of the genus *Bacillus* (Table I). Although a definitive classification to the species level was not possible, based on 16S rDNA gene sequence comparison alone, the closest matches suggested similarity to different *Bacillus* species previously characterized for relevant biotechnological traits. The 16S rDNA sequence of SFB-1 (accession no. DQ988159), SFB-2 (DQ988160) and SFB-3 (DQ988161) shared 99 % identity with *Bacillus licheniformis* (AJ586340), *Bacillus subtilis* (AJ508365) and with *Bacillus* sp. WN613 (DQ275185) (*see* Table I).

Table I. Sequence analysis of partial 16S rDNA (≈737–747 bp) of the culturable aerobic SFB[a] inhabiting the loamy sand cultivated with GM white poplars expressing the *StSy* or *bar* transgenes

Genus	Isolate	Accession no.	*GenBank* closest NCBI database match	Accession no.	Identity, %[b]
Bacillus	SFB-1	DQ988159	*B. licheniformis*	AJ586340	99
	SFB-2	DQ988160	*B. subtilis*	AJ508365	99
	SFB-3	DQ988161	*Bacillus* sp. WN613	DQ275185	99

[a]SFB – spore-forming bacterium(a).
[b]Sequence identity was determined based on the *GenBank* database using BLAST (version 3.0) available at `http://www.dna.affrc.go.jp` and the RDP (Ribosomal Database Project) Classifier (version 1.0) available at `http://www.rdp.cme.msu.edu/`.

DISCUSSION

Our investigation highlighted the lack of significant differences in the size of SFB populations derived from GM and nontransformed white poplars. Thus, no apparent effects on the culturable soil population could be ascribed to transgenic plants. Furthermore, the same response at the soil level was observed with transgenic white poplars expressing transgenes responsible for distinct and unrelated traits, such as resveratrol production and herbicide tolerance. To date, contrasting reports are found concerning the role of SFB as indicators of the environmental impact of GM plants. Cultivation of transgenic *Lotus* plants producing opines did not result in selective pressure on the soil microflora (Oger *et al*. 2000) while significantly higher levels of culturable SFB were associated with transgenic alfalfa expressing lignin peroxidase from *Phanerochaete chrysosporium* (Giovanni *et al.* 1999). More recently, Faragova *et al*. (2005) found higher amounts of SFB in the rhizosphere of GM alfalfa lines engineered with the *Ov* gene from the Japanese quail, encoding a methionine-rich protein ovalbumin.

We cannot exclude that the lack of biological effects observed in the present investigation might be ascribed to the fact that only the soil-culturable SFB fraction was examined. Both microbiological and culture-independent technologies were used by Castaldini *et al.* (2005) in order to search for indicators of the environmental impact of GM Bt corn. The rhizospheric bacterial communities associated with actively growing Bt corn plants and with the GM plant residues in soil were not significantly affected, compared with the nontransformed control. In a recent study (Oliver *et al.* 2008), the effects of transgenic aspen, engineered with a polyphenol oxidase gene, on the rhizosphere bacterial communities have been investigated using cultivation-independent techniques and no significant differences in species diversity were evidenced.

However, since the culturable bacteria correspond to <1 % of the total soil inhabitants (Malik *et al.* 2008), more extensive analysis of the SFB fraction inhabiting the *StSy* and *bar* trials will be required using cultivation-independent methods.

An additional factor, possibly affecting the response of the soil microbial communities, is related to herbicide treatments which were carried out on the *bar* trial (*see* Balestrazzi *et al.* 2008). The investigation performed on the *bar* trial showed that no permanent variations in the size of culturable soil populations occurred after Basta® treatment. Although a detailed study on the SFB was missing, three herbicide-resistant isolates were classified as members of the *Bacillus* genus. The latter, together with the other herbicide-resistant bacteria obtained from the *bar* trial, were not significantly affected by exposure to Basta® treatment (Balestrazzi *et al*. 2008). The impact of glufosinate-tolerant transgenic rice on the soil bacterial communities was investigated by cultivation-based and molecular approaches (Kim *et al.* 2008); also in this case, the bacterial-community structure was mainly affected by soil composition and seasonal changes.

There are discordant reports about the effect of soil temperature on microbial communities; however, the temperature seems to correlate positively with microbial activity, and the temperature changes influence the microbial community structure (Stres *et al*. 2008).

No data are currently available concerning the possible release of resveratrol and/or its derivatives from the GM white poplars into the soil. We have explored the response to purified *trans*-resveratrol in bacteria recovered from leaves of GM white poplars expressing the *StSy* transgene and all the isolates, members of the genus *Bacillus*, were sensitive to *trans*-resveratrol (Balestrazzi *et al*. 2009*a*).

According to current data, the effects observed on microorganisms inhabiting soil cultivated by GM plants are generally transient and associated with specific growth stages of the plant. Some authors also emphasize the fact that the use of multiple approaches might produce ambiguous results (Donegan *et al.* 1999). Some disadvantages related to the studies carried out in contained environment were referred to (Liu *et al.* 2005); however, such studies can offer valuable guidelines to design more accurate field trials.

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