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Soil persistence of DNA from transgenic poplar

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The presence of recombinant DNA in soil cultivated with white poplars (*Populus alba* L.) expressing either the *bar* transgene for herbicide tolerance or the *StSy* transgene for resveratrol production, respectively, was investigated in a greenhouse over a 20-month period. The *bar* trial included the transgenic lines 5P56 and 6EA22P56 and the untransformed line, while the *StSy* trial was established with the transgenic lines 5EAC1 and 12EAC1 and with the untransformed line. All the transgenic poplars harbored the *nptII* marker gene. Plantlets were cultivated in pots, and soil samples were mixed in order to obtain composite pools which were used for molecular analyses. The 35SCaMV-*bar* (1504 bp), 35SCaMV-*StSy* (1403 bp) and NosP-*nptII* (1188 bp) sequences were detected in total DNA extracted from soil samples taken at different times after planting, using PCR/Southern blot hybridization. Microcosm experiments, carried out to assess the effects of temperature and DNA purity on transgene persistence, revealed only a partial correlation between the intensity of hybridization signals and the parameters tested.

Keywords: greenhouse trial / microcosm / *Populus alba* / recombinant DNA / soil persistence

INTRODUCTION

Recombinant DNA released by genetically modified (GM) plants in the soil environment could enhance the frequency of horizontal gene transfer (HGT) from plants to soil microorganisms, causing the undesired spread of transgenes (Monier et al., 2007; Pontiroli et al., 2007). In recent work, HGT events involving bulk and rhizosphere soil bacteria exposed to recombinant DNA were detected at extremely low frequency (Keese, 2008) while the absence of transformable bacteria in rhizosphere- and soil-associated communities suggests that natural transformation represents a rare event (Richter and Smalla, 2007; Van Overbeek et al., 2007).

To date, most of the studies concerning DNA persistence in soil have been carried out using GM annual plants (Pontiroli et al., 2007). Notwithstanding the fact that the exposure of soil-borne microorganisms to the GM material is also dependent on the life-span of the plant, information on forest tree species on this topic is still scanty. Hay et al. (2002) investigated the persistence of the *nptII* marker gene, conferring kanamycin resistance, in decomposing transgenic poplar leaves. The latter were placed in permeable bags and located in a field trial for up to 12 months under different environmental conditions. The recombinant DNA sequences were detected for less than four months. England et al. (2004) used forest litter microcosms spiked with the DNA from a GM baculovirus strain and demonstrated that the target sequence was present for up to three months.

Detection of recombinant DNA in soil microcosms seeded with ground leaf tissue might be affected by factors related to the leaf litter biochemistry which can exert direct effects on the metabolic activities of soil microbial communities and influence the rate of organic matter degradation (Hobbie et al., 2006). Furthermore, it has been reported that plant species with relatively low litter lignin exhibited rapid decomposition rates (Alhamd et al., 2004). The soil microbiological properties during decomposition of *Populus deltoides* leaf litter have been analyzed and compared with *Eucalyptus tereticornis* by Chander et al. (1995), who demonstrated that decomposition of eucalyptus leaves in soils was slower than that of poplar leaves. More recently, Cotrufo et al. (2005) investigated litter decomposition of three *Populus* spp. and observed responses that were strongly species-specific. A more detailed investigation on the dynamics of poplar leaf decomposition in soil focusing on the role of relevant factors still poorly explored, such as the residuesphere (Ceccherini et al., 2003), will help to better assess the fate of recombinant DNA in the soil environment.

The elite cultivar 'Villafranca' (*Populus alba* L.) used in the present study has become a model system in tree biotechnology, and transgenic plants have been produced using, not only conventional gene transfer techniques (Balestrazzi et al., 2006; Giorcelli et al., 2004; Zelasco et al., 2006), but also innovative vector systems for marker-free applications (Zelasco et al., 2007).

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The GM poplar lines 5P56 and 6EA22P56 (Confalonieri et al., 2000) used in this investigation carried a single copy of the *bar* gene from *Streptomyces hygroscopicus* (Thompson et al., 1987), conferring resistance to the herbicide Basta®. The transgenic lines 5EAC1 and 12EAC1 (Giorcelli et al., 2004) carried a single copy of the *StSy* gene from *Vitis vinifera* (Sparvoli et al., 1994) encoding the stilbene synthase enzyme responsible for resveratrol production. Both the *bar* and *StSy* genes were placed under the control of the constitutive 35SCaMV promoter. The GM poplars carried also a single copy of the *nptII* marker gene under the control of the nopaline synthase gene promoter.

PCR and Southern blot hybridization techniques were used to detect the recombinant DNA in total DNA extracted directly from the soil of two experimental trials established with the *bar* and *StSy* GM poplars, respectively, under greenhouse conditions.

Finally, it is worth noting that information concerning the fate of recombinant DNA in soil can be acquired on different scales, and microcosms that mimic the conditions of agricultural soils have been used to monitor DNA persistence and degradation (Lilley et al., 2003; Pontiroli et al., 2007). In the present work, microcosm experiments were carried out with loamy sand in order to investigate the effect of DNA purity and temperature on the fate of the 35SCaMV-*bar*, 35SCaMV-*StSy* and NosP-*nptII* sequences.

RESULTS

Presence of recombinant DNA in soil cultivated with GM white poplars

The trials were planted in the greenhouse in March 2004, using plantlets produced by *in vitro* micropropagation. The experimental design and the sampling scheme of the *bar* and *StSy* trials are shown in Figure 1. Two independent replicates were collected per treatment and sampling point. Total DNA was extracted and the occurrence of the NosP-*nptII*, 35SCaMV-*bar* and 35SCaMV-*StSy* sequences was evaluated by PCR and Southern blot hybridization. For each DNA sample three replicates were used and three independent PCR assays were carried out. When the soil DNA samples were tested in PCR assays with universal bacterial oligonucleotides, the amplification of the 16S rDNA sequence was always observed. The recombinant DNA sequences NosP-*nptII* (1188 bp) and 35SCaMV-*bar* (1504 bp) were detected using oligonucleotide primers which target both the promoter and the associated coding region, in order to avoid the undesired amplification of naturally occurring *nptII* and *bar* genes. The latter were detected in the same soil before GM poplar cultivation, using oligonucleotide primers specific for the *nptII* and *bar* coding region (data not shown).

As for the *bar* trial, samples were first collected in March 2004, in the absence of GM poplars, and molecular analyses performed on the nine pools failed to reveal the presence of the NosP-*nptII* and 35SCaMV-*bar* sequences in the soil environment (Tab. 1, *bar*, 0). After planting, when the average plant height was 30 cm, new samples were taken. It is worth noting that for those soil pools positive to molecular analyses, amplicons were visible only after hybridization with the gene-specific probes. Furthermore, DNA from each pooled sample was amplified three times. Five out of the nine pools from the *bar* field were positive for the PCR/Southern blot hybridization specific for the NosP-*nptII* transgene, while eight out of the nine pools contained the 35SCaMV-*bar* transgene (Tab. 1, *bar*, 3). At six months, the number of NosP-*nptII* positive pools was eight out of nine, while the number of pools carrying the 35SCaMV-*bar* sequence decreased slightly (7/9). Twelve and 18 months after planting (March and August 2005, respectively) the same number (8/9) of positive samples was recorded for both the target sequences.

As concerns the *StSy* trial, no amplification products corresponding to NosP-*nptII* (1188 bp) and 35SCaMV-*StSy* (1403 bp) were detected before planting (Tab. 1, *StSy*, 0). Three months later, approximately eight pools out of twelve were positive in the NosP-*nptII*-specific assay, and six months after planting the number further increased to 10/12 (Tab. 1, *StSy*, 3 and 6). This value remained constant until the end of the experiment (Tab. 1, *StSy*, 6, 12 and 18). As concerns the 35SCaMV-*StSy* region, the number of positive pools was approximately 5/12 and 6/12, three and six months after planting (Tab. 1, *StSy*, 3 and 6). However, 12 months after planting, the fraction of positive pools increased (10/12), and then remained constant until the end of the experiment (Tab. 1, *StSy*, 12 and 18).

The detection limit was assessed with PCR/Southern blot hybridization experiments in which purified poplar DNA extracted from the GM lines was used as template. The intensity of the hybridization signal produced by soil DNA was similar to that obtained using 0.005–0.02 ng of purified poplar DNA as template. Since the poplar diploid genome size is estimated to be 1.12 pg, the detection limit of the system ranged within 5–20 copies of the target sequence.

Persistence of recombinant DNA in soil microcosms

Replicated microcosms carrying purified DNA and ground leaf tissue, respectively, were incubated at 4 °C and 28 °C, for the indicated times. The soil samples

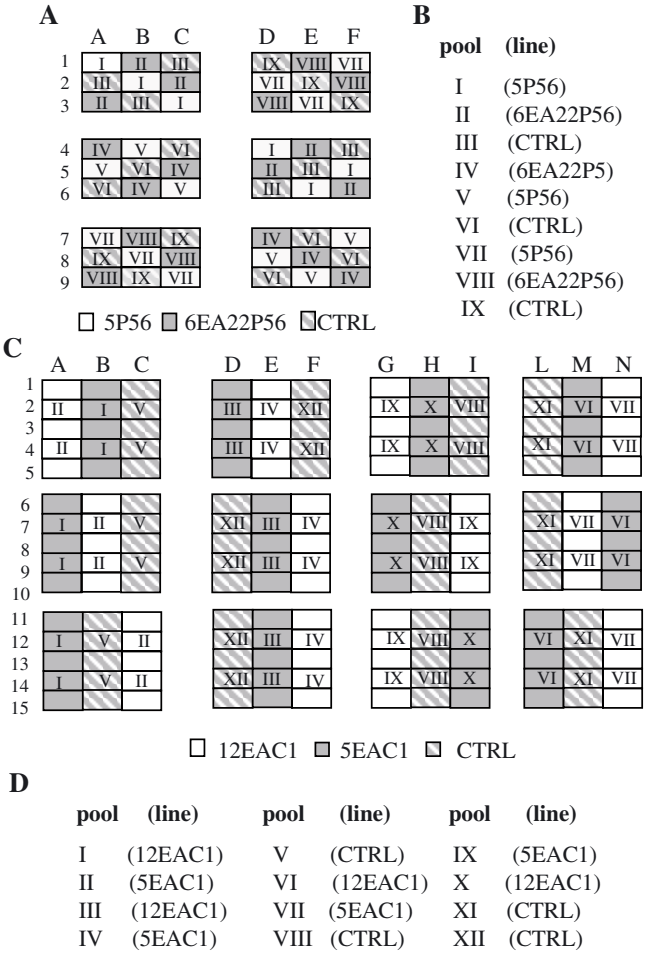

Figure 1. A. Schematic representation of the *bar* trial. Each box represents a single plant belonging to GM line 5P56 (white box), GM line 6EA22 (grey box) and to the untransformed CTRL line (white and gray box). Each plant has been associated with a number (from I to IX) corresponding to a specific soil pool. **B.** List of the nine soil pools obtained from the *bar* trial. Each pool, numbered from I to IX, contained soil collected from six pots cultivated with the same poplar line. **C.** Schematic representation of the *StSy* trial. Each box represents a single plant belonging to GM line 12EAC1 (white box), GM line 5EAC1 (grey box) and to the untransformed CTRL line (white and gray box). Each plant has been associated with a number (from I to XII) corresponding to a specific soil pool. Plants without numbers were not analyzed. **D.** List of the twelve soil pools obtained from the *StSy* trial. Each pool, numbered from I to XII, contained soil collected from six pots cultivated with the same poplar line.

contained either 0.5 g of ground poplar leaf tissue or 500 ng of purified poplar genomic DNA. An average total DNA yield of 20–22.5 µg DNA was recovered from each gram of fresh soil, including the untreated soil samples. PCR assays were carried out using for each sample an equal volume (5 µL) containing 5 ng of total soil DNA. At the beginning of the experiment, the intensity of the hybridization signal produced by all the target sequences was in the range of $1.0–2.0 \times 10^6$ cpm (Fig. 2). As concerns the detection of the 35SCaMV-*bar* sequence in soil maintained at 4 °C, variability was observed in the intensity of the hybridization signals obtained from microcosms seeded with purified DNA and ground tissues throughout the tested period, except for the last time point (10 weeks after the beginning of the experiment), when similar values ($0.7–0.9 \times 10^6$ cpm) were recorded (Fig. 2A, 1). The microcosms containing ground tissue and incubated at 4 °C showed an increased intensity of the hybridization signal at three weeks (1.7×10^6 cpm) (Fig. 2A, 1). At 28 °C, in both types of microcosms there was clearly a time-dependent decrease in the intensity of hybridization signals which ranged from $1.9–2.1 \times 10^6$ to $0.3–0.6 \times 10^6$ cpm (Fig. 2B, 2).

When the 35SCaMV-*StSy* sequence was monitored at 4 °C, opposite responses were observed at one week, as the hybridization signal decreased (from 1.5×10^6

Table 1. Oligonucleotide primer sets used to detect recombinant DNA in soil samples.

Oligonucleotide	Sequence	Amplicon size (bp)
Nos-1	5′-GAGCGGAGAATTAAGGGAGTCA-3′	NosP-*nptII*
K-3	5′-GGCGATAGAAGGCGATGCGCTG-3′	1188
35S-FW1	5′-CAGAAAGAATGCTAACCCACAGA-3′	35SCaMV-*bar*
BAR-3	5′-CAGATCTCGGTGACGGGCAG-3′	1504
35S-FW2	5′-CAGTGGTCCCAAAGATGGACC-3′	35SCaMV-*StSy*
StSy-2	5′-TTAATTTGTCACCATAGGAATGCTA-3′	1403
K-1	5′-AGGCTAATTCGGCTATGACTGG-3′	*nptII*
K-2	5′-GCGGTCCGCCACACCCAGCCG-3′	549
BAR-1	5′-TCCCCCGGGGGAATGAGCCCAGAACGACGCCC-3′	*bar*
BAR-2	5′-CGAGCTCGTCAGATCTCTGTGACGGGCAG-3′	652
StSy-1	5′-ATGGCTTCAGTCGAGGAAATTAG-3′	*StSy*
StSy-2	5′-TTAATTTGTCACCATAGGAATGCTA-3′	1178

Table 2. Detection of recombinant DNA sequences in loamy sand cultivated with transgenic white poplars. PCR analyses were carried out using gene-specific oligonucleotide primers that allowed the amplification of DNA fragments spanning both the promoter and the coding region.

	Experimental trial			
	bar		*StSy*	
Months after planting	35SCaMV-*bar*[a]	NosP-*nptII*	35SCaMV-*StSy*	NosP-*nptII*
0	n.d.[b]	n.d.	n.d.	n.d.
3	8/9	5/9	5/12	8/12
6	7/9	8/9	6/12	6/12
12	8/9	8/9	6/12	6/12
18	8/9	8/9	6/12	6/12

[a] N° positive pools/N° total pools; [b] not detected.

to 1.0×10^6 cpm) in microcosms seeded with purified DNA, and increased (from 0.9×10^6 to 1.5×10^6 cpm) in those samples containing ground leaf tissue (Fig. 2A, 3). When the microcosms were incubated at 28 °C, no differences were observed in the intensity of hybridization between the two treatments (Fig. 2B, 4). In microcosms diluted with purified DNA and incubated at 4 °C, the amount of the NosP-*nptII* PCR product showed an enhancement at one week (from 0.8×10^6 to 2.6×10^6 cpm), while in microcosms containing the ground tissue, a peak in the hybridization signal (1.4×10^6 cpm) was observed at three weeks. The detection patterns observed at 28 °C were quite similar for both types of microcosms, and ranged from $1.8–1.9 \times 10^6$ cpm to $0.4–0.8 \times 10^6$ cpm (Fig. 2B, 6).

The efficacy of the detection system was assessed by PCR/Southern hybridization experiments carried out using as template diluted amounts of purified poplar genomic DNA extracted from the 5P56 and 12EAC1 GM lines. The intensity of the NosP-*nptII* hybridization signal produced by PCR amplification of 0.02 ng of purified poplar DNA was similar to the highest NosP-*nptII* hybridization signal obtained in Figure 2B (week 0, purified DNA). In this specific case, the system was highly effective (close to 100%) in monitoring the recombinant DNA in soil, since the estimated amount of poplar DNA in each tube corresponded to 0.027–0.031 ng. The efficacy was reduced in other cases.

DISCUSSION

The investigation carried out with the *bar* and *StSy* trials showed that detectable amounts of the recombinant DNA were present in the soil. The increasing proportion of the total root system that developed in each

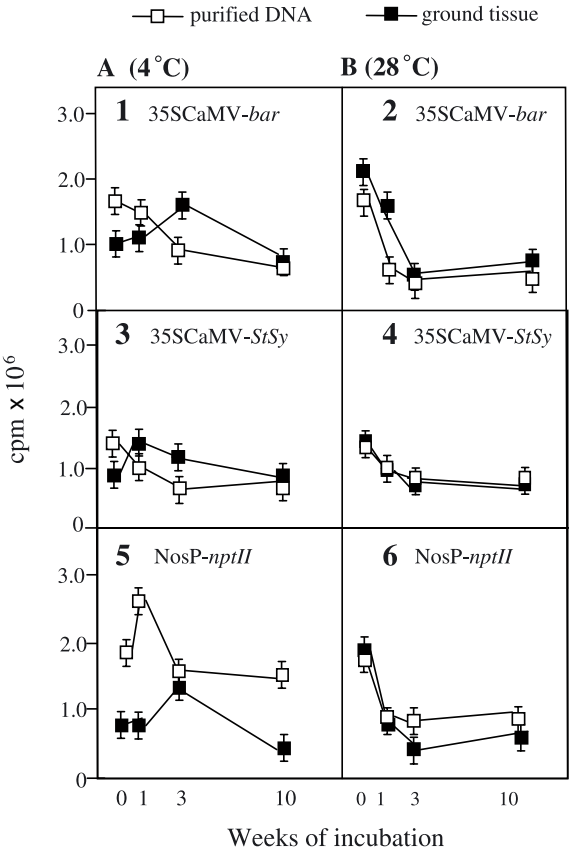

Figure 2. Detection of recombinant DNA sequences in soil microcosms containing purified DNA (□) and ground leaf tissues (■) from transgenic white poplars. PCR assays were carried out using gene-specific oligonucleotides and the resulting amplification products were hybridized with gene-specific probes. The intensity of hybrization signals, expressed as counts per minute (cpm) is shown. **A.** Microcosms were incubated at 4 °C for 0, 1, 3 and 10 weeks. **B.** Microcosms were incubated at 28 °C for 0, 1, 3 and 10 weeks.

pot might explain the higher frequency of positive PCR assays obtained for the pooled samples. The dynamics of spread of recombinant DNA by roots during potato plant growth has been analyzed by de Vries et al. (2003), who carried out both field plot and greenhouse experiments. These authors detected the recombinant *nptII* sequence in soil collected from non-transformed potato plants grown in close proximity of GM plants. This was due to the presence of roots from transgenic plants that spread rapidly, reaching the neighboring plants. De Vries et al. (2003) remarked that roots can release DNA in soil during plant growth, either as a free molecule or within plant tissue material, due to the *in situ* destruction of rhizodermis or root cap cells. The finding that also those pools derived from soil cultivated with untransformed plants contained recombinant DNA might be due to contamination occurring during DNA extraction and purification, although spread of GM material caused by the accidental detachment of GM leaves in the greenhouse could not be excluded. Other biological processes, such as those involving microbial nuclease activities, might have affected the integrity of recombinant DNA in the loamy sand. Although in previous work (Balestrazzi et al., 2007), fluctuations were observed in the size of the culturable bacterial fraction able to secrete nucleases and inhabiting the soil of the *bar* trial, the complex interactions between the soil particles and nucleic acids (Blum et al., 1997) need to be considered.

It is worth noting that discrepancies were observed in the response of soil pools obtained from the poplar trials, since some of them were unexpectedly negative. The non-homogeneous distribution of humic acid and other inhibitors in the soil DNAs might represent a possible explanation for this negative result, since it has been reported that oligonucleotides designed to detect different DNA regions might have different behavior when added to DNA samples with impurities (Krsek and Wellington, 1999).

The presence of 35SCaMV-*bar*, 35SCaMV-*StSy* and NosP-*nptII* sequences was also evaluated in microcosms in the attempt to define the effects of soil temperature and DNA purity on transgene persistence. It is expected that at increasing temperatures the soil microbial activity and particularly the nuclease activity are enhanced, thus affecting the integrity of free DNA (Luo et al., 2001). Experiments were designed according to Widmer et al. (1997), who performed a microcosm study with purified plasmid DNA added to soil and also tested a mixture of soil and ground fresh leaf tissue from transgenic tobacco plants. In the present work, persistence of recombinant DNA in soil microcosms was monitored for approximately three months and the target sequences were always detected. In a different report, Gebhard and Smalla (1999) monitored the persistence of DNA extracted from transgenic sugar beet plants and added to soil microcosms. The purified DNA was mixed with soil and incubated at 20 °C for different time periods. PCR detection of the *nptII* and *bar* genes and their associated promoter regions resulted in the presence of positive signals up to six months after the beginning of the microcosm experiment. They also found that the intensity of the hybridization signal decreased during the first day and subsequently increased. According to these authors, different parts of the constructs may be differently accessible, due to the secondary structures or interactions with the plant genome. In our hands, all the hybridization signals produced by soil microcosms incubated at 28 °C and two out of the six hybridization bands obtained at 4 °C decreased one week after the beginning of the experiment,

and the intensity of signals further decreased during the subsequent time point (three weeks after the beginning of the experiment). Due to the different time period investigated, it is difficult to compare our data with those from sugar beet microcosms.

The semi-quantitative approach used with the recombinant DNA sequences in white poplar microcosms revealed only a partial correlation between the intensity of hybridization signals and the tested parameters (DNA purity and soil temperature). In recent work, Lerat et al. (2005) reported on the quantification of recombinant DNA from GM corn and soybean in soil samples by real-time PCR, and remarked the difficulties in performing amplification due to the presence of co-extracted humic acids. This might represent a possible explanation for the data obtained with our soil microcosms. However, it is worth noting that spectrophotometric analyses carried out to assess the degree of humic acid and protein contamination revealed low levels of these substances (Balestrazzi et al., unpublished results).

MATERIALS AND METHODS

Soil description

The agricultural soil used in this study was collected from cultivated fields surrounding the town of Lodi, in the southern part of Lombardy. The soil was mixed with white peat (20%; Tecnic, Free Peat B. V., The Netherlands) in order to facilitate the rooting of the poplar plantlets. The resulting substrate was classified as a medium-textured loamy sand (77.43% sand, 16.93% silt and 5.62% clay; 24.58% organic matter; 62.0% Water Holding, Cation Exchange Capacity of 3.32 meq.g^{-1} with pH 6.85). The organic C content and the total N content were estimated 3.02% and 0.256%, respectively, which corresponded to a C:N ratio of 11.79.

Plant materials

The plant materials, produced by *in vitro* micropropagation, was transferred into pots and then acclimatized for four weeks in a growth room at 22 °C under a photoperiod of 16 h, a photon flux density of 150 μmol.m^{-2}.s^{-1} and a relative humidity of approximately 70–80%. After this period, all the rooted plantlets were transplanted into pots (30 cm diameter, 34 cm height) containing fresh soil and placed in the greenhouse under equal light intensity and temperature. Plants were supplied with a nutrient solution containing N (168 mg.L^{-1}), P (46.5 mg.L^{-1}), K (237.7 mg.L^{-1}), Na (230 mg.L^{-1}), Ca (200 mg.L^{-1}), Mg (24.3 mg.L^{-1}), S (81.7 mg.L^{-1}), Fe (14 mg.L^{-1}), Mn (1.7 mg.L^{-1}), B (2.1 mg.L^{-1}), Zn (1.4 mg.L^{-1}), Cu (0.18 mg.L^{-1}), E.C. = 1.9 mS.cm^{-1}, pH 6.0.

Experimental design

The *bar* trial consisted of 54 poplars (18 plants for each line) while the *StSy* trial was established with 180 poplars (60 plants for each line) (Figs. 1A and 1C). For the analyses carried out on the greenhouse trials, soil samples were collected at a depth of 0–18 cm from each pot, using a Soil Auger Bucket (Soilmoisture Equipment Corp.). The diameter of the auger was 3 cm. Samples were then transferred to sterile Magenta boxes (77 mm × 77 mm × 97 mm, Sigma Aldrich), maintained at 4 °C, immediately transported to the laboratory and used less than 2 h after removal. In the *bar* trial, samples from six different pots were mixed in order to obtain nine composite samples (pools). In the *StSy* trial, twelve composite pools were obtained. According to this sampling scheme, described in Figures 1B and 1D, each pool contained soil collected from six pots cultivated with the same poplar line. Two independent replicates (composite soil samples) per treatment and sampling point were collected.

Collection of leaf tissues and DNA extraction

For microcosm experiments, leaves were collected from six-month-old poplar plants during the vegetative growth phase, and immediately frozen in liquid nitrogen. Genomic DNA was extracted from the ground leaves as described by Rogers and Bendich (1988), and concentration evaluated by UV spectrophotometry and agarose gel electrophoresis (Sambrook et al., 1989).

Microcosm design

Soil aliquots (2 g) were transferred to 50-mL sterile tubes and intensively mixed with equal amounts of purified genomic poplar DNA extracted from the 5P56 and 12EAC1 lines, respectively (250 ng for each GM line). Thus, 4.46×10^5 poplar genomes were present in a single tube. This corresponded to 4.46×10^5 copies of the *nptII* marker gene and 2.23×10^5 copies of the *bar* and *StSy* transgene, respectively. The same experiment was performed by adding 0.5 g (fresh weight; 0.25 g from each GM line) of ground poplar leaf tissue to single tubes containing 2 g of soil. Sterile distilled water was added to the soil to reach a final moisture content of 40% (v/wt). The microcosms were incubated at 4 °C and 28 °C, respectively. Two independent experiments were carried out, and for each treatment three different tubes were used as replicates.

Soil DNA extraction and purification

DNA extraction from soil samples was carried out using the high-salt, SDS-based, extended-heating method

of Zhou et al. (1996). Crude DNAs were subsequently purified using the GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences) as follows: each crude DNA sample (12.5–25 μL) was loaded onto the column, the adsorbed DNA was washed and collected according to the protocol supplied. This step was then repeated and finally the purified DNA was collected and stored at −20 °C. DNA concentration was evaluated by agarose gel electrophoresis (Sambrook et al., 1989).

PCR analyses

All PCR reactions were carried out in a final volume of 30 μL containing 0.2 mM premixed deoxynucleoside triphosphates (M-Medical S.r.l.), 1.5 mM $MgCl_2$ and 2.5 U *Taq* DNA Polymerase (DyNAzyme II, Finnzymes, Celbio), using a T Gradient apparatus (Biometra). In order to detect the presence of the recombinant DNA sequences, PCR analyses were carried out using three different oligonucleotide sets (Tab. 1). Three independent PCR assays were carried out, and for each DNA sample three replicas were used. Amplification was carried out as previously described at 94 °C for 50 s, 62 °C for 50 s, 72 °C for 2 min (35 cycles). PCR products were separated on 0.8% (w/v) agarose gels (Duchefa Biochemicals) and then blotted to membranes (Hybond™-N^+, Amersham Biosciences) according to the supplier's suggestions.

Molecular probes and Southern blot hybridisation

The gene-specific probes, containing the *nptII*, *bar* and *StSy* coding regions were obtained by PCR as previously described, using the gene-specific primer sets shown in Table 1. Amplification was carried out at the following conditions: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min (35 cycles) for the *nptII* sequence, 94 °C for 50 s, 62 °C for 50 s, 72 °C for 50 s (35 cycles) for the *bar* gene, 94 °C for 50 s, 62 °C for 50 s, 72 °C for 1 min and 30 s (35 cycles) for the *StSy* gene. PCR products were separated on 0.8–2.0% (w/v) agarose gels (Duchefa Biochemicals) and purified from agarose gels using the GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences). Sequence analysis was performed using an ABI 3730XL apparatus (Applied Biosystems). Each gene-specific probe was labeled with α-[^{32}P]-dCTP using the Hexa Label Plus™ DNA labeling kit (M-Medical S.r.l.). Filters were hybridized under the following conditions: 50% (v/v) formamide, 5 X SSC (150 mM NaCl, 15 mM Na3 citrate pH 7.6), 0.5% SDS, 5 X Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 100 $\mu g.mL^{-1}$ salmon sperm DNA at 42 °C for 16 h. Final washes were performed under high stringency (0.1 X SSC/0.1% SDS, at 65 °C for 10 min). Densitometric analysis was performed using a Biostep GmbH apparatus with the argus X1 3.3.0 software.

Statistical analyses

Statistical analysis was carried out using the Microsoft® EXCEL2000 (9.0.2812) statistical package calculating mean and standard error. 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).

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