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Occurrence of Culturable Bacteria Living in Micropropagated Black Locust Cultures (*Robinia pseudoacacia* L.)

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Summary

The culturable bacteria living as associated and endophytic microorganisms in tissue cultures of black locust selected randomly from roots as well as shoots were characterized. During a ten week cultivation period, the population density increased by approximately 10^2 to 10^3, and showed the highest density in the root tissue by approximately 10^8 to 10^9 cfu g^{-1} fresh weight. Sequencing of the 16S rRNA gene of 119 isolated strains revealed 20 different genotypes belonging to six genera from the phyla *Actinobacteria* and *Proteobacteria.* The *Actinobacteria* which represented the majority of isolates were divided into up to 12 16S rDNA genotypes belonging to the genera *Microbacterium* and *Curtobacterium*. The *Proteobacteria* (8 genotypes) was comprised of the genera *Sphingomonas* (2 genotypes), *Methylobacterium* (1 genotype), *Acidovorax* (1 genotype), and *Dyella* (4 genotypes).

Key words. *Robinia pseudoacacia* – endophytic bacteria – 16S rDNA – tissue culture

Introduction

Bacterial endophytes have been reported in various plant tissues. Similarly, micropropagated plantlets are not sterile organisms as endophytic bacteria can persist in a latent form within the sterile *in vitro* culture system (Bastiaens 1983).

During plant propagation by *in vitro* methods, the enrichments of endophytic bacteria were frequently observed in cultures of forest tree species, such as Scots pine (Pirttilä et al. 2004), Norway spruce (Ewald et al. 1997) as well as black locust (Naujoks et al. 2000).

Black locust is of increasing interest for the timber industry as it is a fast growing species that produces a hard and durable wood. The tree is characterized by efficient growth on poor and nitrogen-deficient soils and is suitable for afforestration of arable land, as well as in devastated areas. For this reason, selected clones of black locust possessing outstanding growth properties combined with straight trunks are frequently propagated *in vitro*. During micropropagation the occurrence of bacteria were observed regularly. Their action ranged from no influence to complete failure of regeneration. Because a contamination had been excluded from outside those bacteria were assigned to the group of endophytic living organisms. In a previous study single isolates were classified according to the BIOLOG and API identification systems as *Curtobacterium* sp. (Naujoks et al. 2000) but other strains could not identified with these methods so far. The objective of this study was to get more knowledge on the number of the endophytic bacteria as well as their community structure of symptomless *in vitro* regenerated rooted plants of *R. pseudoacacia*.

Material and Methods

Plant material

Four micropropagated clones of *R. pseudoacacia* named 2439*new*, 2439*old*, 2802*old* and 2494*old* were used. The clone 2439*new* was cultured *in vitro* for two years and was re-established from clone 2439*old,* cultured *in vitro* for four years. The clones 2802*old* and 2494*old* have been micropropagated continuously for nine and for fourteen years, respectively. The plant material was propagated and rooted according to Naujoks et al. (2000).

The mericloning of axillary tissue was followed by micropreparation of axillary meristems under a microscope and got transferred onto $^1/_2$ concentrated Murashige & Skoog-medium (Murashige and Skoog 1962). The medium was enriched for cultivation of meristematic tissue with 6-benzylaminopurine (0.15 mg L^{-1}), α naphthalene acetic acid (0.001 mg L^{-1}), gibberellic acid A3 (0.5 mg L^{-1}), kinetin (0.03 mg L^{-1}), and sucrose (20 g L^{-1}).

Cultures were grown for ten weeks before transplanting. The light quantum was 25–60 μE m^{-2} s^{-1} for the first five weeks period and 35–60 μE m^{-2} s^{-1} for the second period

Determination of the bacterial population density and screening of isolates

The titre of the endophytic bacteria was estimated from each five plantlet replicates of the clones 2439*old* and 2802*old.* Root systems, shoot axis with developed leaflets, and shoot apex were sampled separately and their

fresh weights were measured. Plant parts were surface-disinfected in 0.25 % $HgCl_2$, rinsed three times in sterilized water, crushed and shaken in 80 ml sterile tap water for five hours. Three serial dilutions per plant part were prepared and 100 µl aliquots were plated threefold on Yeast extract-Mannitol Agar (YMA) (ELKAN and BUNN 1992). The plates were incubated at 28 °C for three days.

Bacteria densities were measured at three time points starting four weeks after transfer to rooting medium, after five, and after 10 weeks later. The data were related to fresh weight. Additionally, the population density was determined of shoots of the clones 2498*new* and 2439*old* which were regenerated from meristematic tissue of shoots axis.

Selection of endophytic bacteria was carried out from roots and total shoot axis separately using all four clones. Sterile plant material was treated as described and plated on YMA, KBBC medium K5120 (proteose peptone 20 g L^{-1}, K_2HPO_4 1.5 g L^{-1}, H_3BO_3 1.5 g L^{-1}, $MgSO_4$ anhydrous 0.73 g L^{-1}, agar-agar 20 g L^{-1}, DUCHEFA, The Netherlands) and *Methylobacteria* medium (http://www.dsmz.de/media/media.htm) in three replicates. Colonies differed in their appearance like growth and colour were picked randomly and were further subcultured on YMA. Additionally, some isolates were selected from the plantlets without surface sterilization. Those strains were designated as plant-associated bacteria.

Data of population densities were analyzed using BOXPLOT procedure by SAS V9.12 (SAS Institute Inc., Cary, NC, U.S.A.) and computed with ANOVA using PROC MIXED procedure.

Classification of isolates

Fresh bacterial cells were washed in sterile 0.3 % NaCl, resuspended in 200 µl lysis buffer (25 mM NaOH/0.25 % SDS) and incubated by shaking for 15 min at 95 °C. The lysate was mixed with 100 µl of 3 M sodium acetate (pH 5.2) and centrifuged. An equal volume of isopropanol was added to the supernatant, centrifuged at 4 °C for 10 min, the pellet was washed with 70 % ethanol before being dissolved in 50 µl TE buffer.

The amplification of the 16S rRNA gene was carried out using the primers fd1 (5'-AGA GTT TGA TCC TGG CTC AG-3') (WEISBURG et al. 1991) and 926r (5'-CCG TCA ATT CCT TTR AGT TT-3') (MUYZER et al. 1993). A 50 µl reaction mixture contained 1 µl DNA template, 5 µl 10 x PCR buffer, 3.5 µl 25 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µl of each primer (100 pmol μl^{-1}) and 0.5 µl Taq polymerase (Applied Biosystems). Amplifications were performed in a GeneAmp®-PCR System 9700 (Applied Biosystems) using the following protocol: initial denaturation at 95 °C for 2 min, 25 cycles of 30 s at 94 °C, 40 s at 54 °C and 1.5 min at 72 °C; followed by a final hold at 4 °C.

The PCR products were visualized in a 1 % agarose gel and purified by the Invisorb®Spin PCRapid Kit (Invitek, Berlin, Germany). Concentrations of DNA were measured using a spectrophotometer (Nanodrop, Novell, Wilmington, U.S.A.). DNA sequencing was performed using a BigDye terminator cycle sequencing Kit (Applied Biosystems) and the primer 346r (5'- TGT AGT CCT GGT GCA GGC TCT T -3') (CHUN and GOODFELLOW 1995) on an AbiPrism™ 310 (Applied Biosystems). The partially sequenced 16S rDNA fragments were compared to sequences available from the Ribosomal Database Project (RDP) and EMBL/GenBank. The similarity values were based on a pair-wise sequence comparison. The assignment of the isolates at the genus level was based on the BLAST analysis and on the taxonomic assignment by the Classifier program of the RDP. For phylogenetic analyses, DNA sequences were aligned using the Clustal W algorithm (THOMPSON et al. 1994); afterwards phylogenetic trees were generated using the program MegAlign (Dnastar) (www.dur.ac.uk/stat.web/Bioinfofrmatics/megalign.htm).

Partially sequenced 16S rDNA genes derived from 17 isolates were deposited in NCBI Genbank (acc. no. DQ358210–DQ358226).

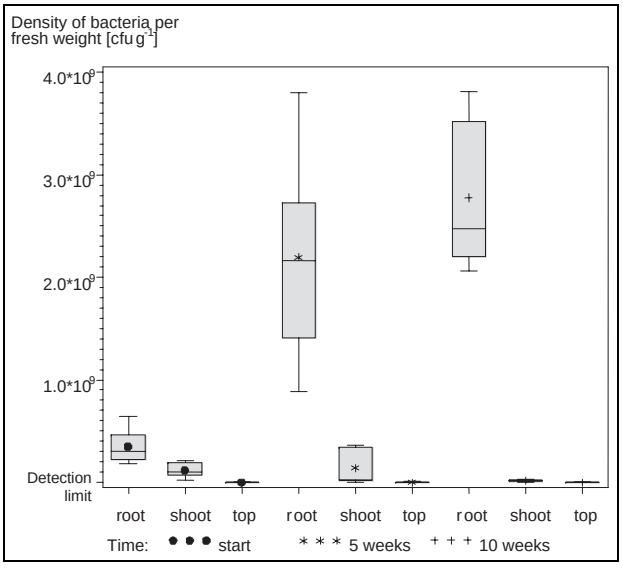


Fig. 1. Boxplots of endophytic population density estimated in different compartments of the black locust clone 2494*old* at three different time points.

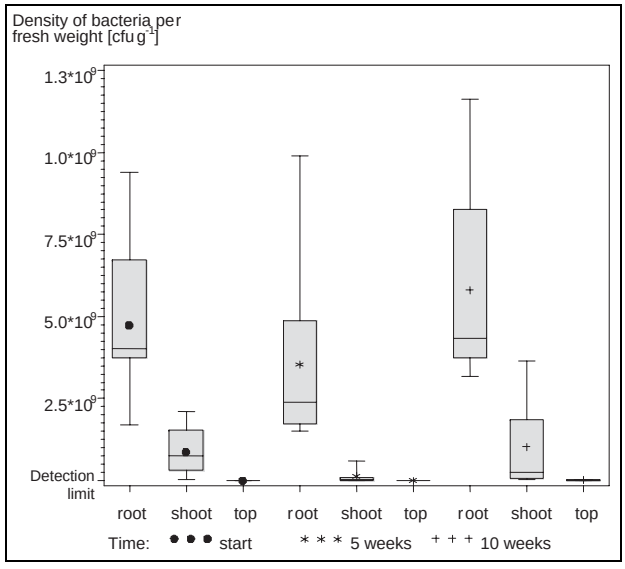


Fig. 2. Boxplots of endophytic population density estimated in different compartments of the black locust clone 2802*old* at three different time points.

Results

The population densities of endophytic bacteria of clones 2439*old* and 2802*old* differed between the three plantlet parts investigated. The roots showed congruently highest number of culturable bacteria (Fig. 1, 2) which were significantly different ($P<0.05$) from those of the shoots and tips, independently from cultivation time.

An increase of bacteria titres was observed in the roots of clone 2494*old* during the 10 weeks cultivation period (Fig. 1), whereas the roots of clone 2802*old* showed no changes during this time (Fig. 2). The mean numbers of bacteria within roots were 2.8 x 10^9 cfu g^{-1} fresh weight of clone 2494*old* and 5.8 x 10^8 cfu g^{-1} of clone 2802*old* at the end of observation period. The population densities of shoots were lower and showed no, or only slight changes. The mean values ranged from 1.5 x 10^7 cfu g^{-1} of clone 2494*old* to 1 x 10^8 cfu g^{-1} of clone 2802*old*. The data for the shoot apex were low and ranged near the detection limit (6 x 10^3 cfu g^{-1}).

The numbers of bacteria obtained from regenerated shoots from axillary meristem differed severely between the two clones tested and amounted 6 x 10^5 cfu g^{-1} fresh weight for the clone 2498*new* and 2.5 x 10^8 cfu g^{-1} for the clone 2439*old*. The mean numbers increased to 4.6 x 10^8 cfu g^{-1} (clone 2498*new*) and 2 x 10^9 cfu g^{-1} (clone 2439*old*) at the end of the cultivation period. (data not shown)

Analysis of randomly selected isolates from all four clones revealed a diverse bacterial community. Sequencing of 16S rDNA fragments of 119 isolates resulted in 20 genotypes (Table 1) belonging to six genera of the two phyla *Actinobacteria* and *Proteobacteria.* Phylogenetic analysis is demonstrated in Fig. 3.

The majority of isolates (97) were members of the family *Microbacteriaceae,* representing 12 different genotypes. *Proteobacteria* were less represented with only 22 isolates. They were divided into eight different genotypes belonging to α-, ß-, and γ-*Proteobacteria*.

The classification of the isolates showed no indication of the presence of tissue-specific groups for shoots and roots. Furthermore, there was no specificity of isolates of certain bacterial genera for one of the tested black locust clones.

Within total isolates, the genus *Microbacterium* was predominant (96 isolates) with eight different genotypes (types 2.0, 2.1a, 2.1b, 2.2–2.8). The majority was closely related to *M. flavescens* (strain IFO15039^T). Two strains retrieved from the outer shoot (type 2.4 and 2.5) were closely related to *M. trichotecenolyticum*. The genotypes 2.7 and 2.8 seemed to be distantly related within that group and showed only a relatively low similarity to *M. flavescens* (97 and 96 %, resp.). Another two genotypes belonging to *Microbacteriaceae* were assigned to the genus *Curtobacterium* (types 5.0a and 5.0b isolated from the clones 2494*old* and 2802*old*). Four genotypes (types 3.0, 3.0a, 3.1, 3.2) with eight isolates belonging to γ-*Proteobacteria* were identified as strains closely related to *Dyella yeojuensis.* Types 3.0, 3.0a and 3.1 differed in only one nucleotide from the sequence of the *D. yeojuensis* strain R2A16-10. Type 4.1 were found to be most related to the genus *Sphingomonas* with the highest similarity to the not specified more closely strain CanClear1. Type 6 belonging to the α-*Proteobacteria* showed a relatedness to

Table 1. Genotypes of endophytic and plant-associated bacteria isolated from *in vitro* cultures of black locust.

Type no.	Clone	Habitat	No of isolates	Closest relatedness to strain of GenBank	Homology
1.0	2439*old*	shoot a[1]	1	*Acidovorax defluvii* strain BSB411, Y18616	99 %
2.0	2349*old*	shoot a	1	*Microbacterium flavescens* strain IFO15039T, AB004716	99 %
2.1a	2439*old*	shoot e[2]	65	*Microbacterium flavescens* strain IFO15039T, AB004716	99 %
2.1b	2439*old*	root e	13	*Microbacterium flavescens* strain IFO15039T, AB004716	99 %
2.2	2439*old*,	shoot e	2	*Microbacterium flavescens* strain IFO15039T, AB004716	98 %
2.3	2439*old*	root e	10	*Microbacterium flavescens* strain IFO15039T	99 %
2.4	2439*old*	shoot a	1	*Microbacterium trichothecenolyticum* AB167383	99 %
2.5	2802*old*	shoot a	1	*Microbacterium trichothecenolyticum* AB167383	98 %
2.6	2439*new*	shoot e	1	*Microbacterium flavescens* strain IFO15039T, AB004716	98 %
2.7	2439*new*	shoot e	1	*Microbacterium flavescens* strain IFO15039T, AB004716	97 %
2.8	2439*new*	shoot e	1	*Microbacterium flavescens* strain IFO15039T	96 %
3.0	2439*old*	shoot a	1	*Dyella yeojuensis* strain R2A16-10, DQ181549	99 %
3.0a	2439*old*	shoot e	5	*Dyella yeojuensis* strain R2A16-10, DQ181549	99 %
3.1	2439*old*	root e	1	*Dyella yeojuensis* strain R2A16-10, DQ181549	99 %
3.2	2494*old*	shoot a	1	*Dyella yeojuensis* strain R2A16-10, DQ181549	98 %
4.0	2439*old*	shoot a	1	*Sphingomonas* sp. strain CanClear1, AF395038	99 %
4.1	2439*old*	shoot e	2	*Sphingomonas* sp. strain CanClear1, AF395038	99 %
5.0a	2494*old*	shoot a	1	*Curtobacterium citreum* strain CC-88221, AY961986	99 %
5.0b	2802*old*	shoot a	1	*Curtobacterium citreum* strain CC-88221, AY961986	99 %
6.0	2439*old*	shoot e	9	*Methylobacterium fujisawaense* DSM 5686, Z23160	98 %

[1]a = plant-associated
[2]e = endophytic

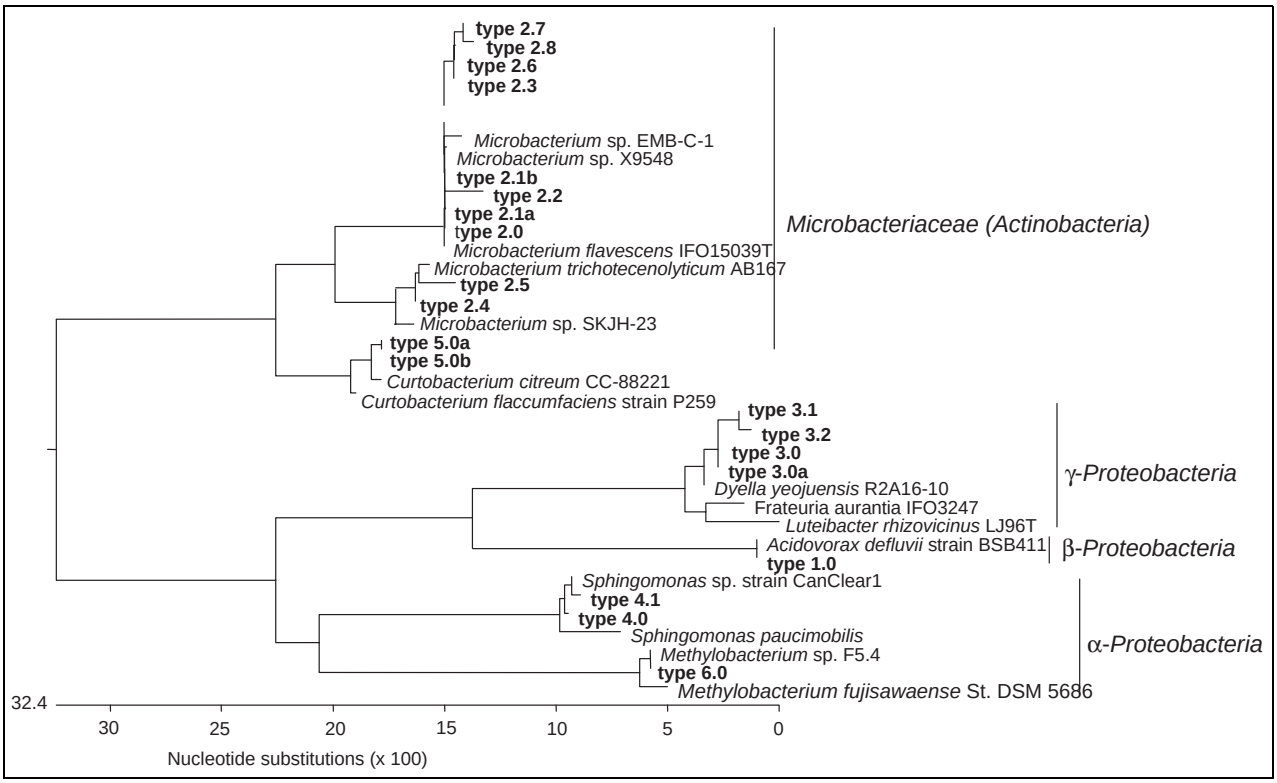


Fig. 3. Phylogenetic tree showing the relationships of isolates from micropropagated black locust cultures with selected reference strains.

the genus *Methylobacterium* with a similarity of 98% to *M. fujisawaense* DSM 5686.

Discussion

This study provides the first report on the population densities of culturable endophytic and plant-associated bacteria in *in vitro* cultures of black locust clones. Results showed a phylogenetic diversity with six genera of two different phyla. In both culture types (callus and meristem cultivation) an accumulation and spread of dominant bacteria could be observed during a ten weeks cultivation period.

All 20 genotypes identified were related to common endophytic and plant-associated bacteria, as well as to soil and water-inhabiting strains. Most of the endophytic isolates were assigned to *Microbacterium* with a majority similar to *M. flavescens*. This frequency measured under *in vitro* conditions may give an advice of a beneficial functional interaction between black locust plants and *Microbacterium* strains. *Microbacterium* species were detected on grasses like *Agropyron* (Behrendt et al. 2002) as a typical member of phyllosphere communities.

Another genus of the found endophyte complex was *Curtobacterium* with genotypes similar to *C. citreum*. This species had been isolated as an endophyte from several woody plants like elm, sweet-orange, and coffee, (Araújo et al. 2002; Mocali et al. 2002; Vega et al. 2005). This genus had already found in a previous study with black locust (Naujoks et al. 2000).

Furthermore, some isolates have been proven to belong to different classes of *Proteobacteria*. Within the genus *Sphingomonas* nitrogen fixing species exist and they are considered to be soil and plant-associated. In poplar, *Sphingomonas* was the most abundant genus with about 24 % (Ulrich et al. 2007). The species most closely related to isolates from black locust is *Sphingomonas paucimobilis* strain ATCC29837, which was already found in grasses, cotton, corn, and rice (McInroy and Kloepper 1996).

The genus *Methylobacterium* features a range of species ubiquitously associated with the phyllosphere. Some species belong to the plant growth promoting bacteria producing phytohormons and have antagonistic properties (Omer et al. 2004). Other members of that genus were found in poplars (Van Aken et al. 2004) and pines (Pirttilä et al. 2004).

The class of ß-*Proteobacteria* is represented by an *Acidovorax* species similar to *Acidovorax defluvii* strain BSB411 which was detected in activated sludge (Schulze et al. 1999). Members of this genus were found to be associated with roots of grasses and from rhizosphere (Wang and Skipper 2004).

The genotypes of the genus *Dyella* (γ-*Proteobacteria*) are related to the species *Dyella yeojuensis* which have been proven as endophytes of pine and coexisted in a community together with white pine ectomycorrhizas (Izumi et al. 2007).

Generally, the found composition of the bacterial community within plants depends on the choice of methods. In this study, only culturable bacteria were investigated

isolated with standard growth media. This part of the endophytic and plant-associated community of black locust might differ from that obtained by culture-independent methods as a cloning approach of 16S rRNA genes. The appearance of soil, water, and plant-inhabiting bacteria, as well as colonizers of the rhizosphere living endophytically in tissue cultures of black locust clones would suggest an interaction with adjacent bacterial habitats in the natural environment of the mother trees. However, the knowledge about the role and the mode of action of the endophytic bacteria is limited and remains to be addressed by future investigations.

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