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ORIGINAL PAPER

Isothermal DNA amplification facilitates the identification of a broad spectrum of bacteria, fungi and protozoa in *Eleutherococcus* sp. plant tissue cultures

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Abstract In vitro cultures of *Eleutherococcus sieboldianus* originating from surface sterilized leaf explants were found to be associated with several microorganisms. The associations included bacteria, fungi and protozoa within the rhizosphere and inside root hairs. To determine if this phenomenon is unique to this species, plant tissue cultures of *E. gracilistylus* and *E. senticosus* were included in our studies for comparison. A methodology consisting of isothermal amplification, cloning and sequencing was established for analysing 16*S* ribosomal DNA of cultivated and non-cultivated bacteria from different tissue types. The same methodology was used to obtain internal transcribed spacer regions and 18*S* regions of fungal and protozoan rDNA. Comparative analyses of sequencing data resulted in the identification of various genera within the Firmicutes and γ-proteobacteria kingdoms and a broad spectrum of fungal genera related to several uncultured fungi. In addition, amoebal and chrysophyte species were detected. Most of the species were identified in different plant organs and in in vitro culture cell types indicating the microorganisms are systemically distributed. The presence of identical microorganisms in different plant species argues for an evolutionary long-lasting and stable association between the plant genus and the microinhabitants.

Keywords *Acanthopanax* (Araliaceae) · Root hair endophytes · Non-cultured microorganisms · Internal transcribed spacer region (ITS) · Phi-29 DNA polymerase and isothermal DNA amplification

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Introduction

Different representatives of the plant genus *Eleutherococcus* (Syn. *Acanthopanax*) of the Araliaceae family play important roles in medicine, in agriculture and as a source of food. These shrub-like plants naturally occur in locations in Asia (China, Vietnam, Korea, Japan) (Kim 1997). *Eleutherococcus sieboldianus* a native of Japan is cultivated as a solitary plant or as hedges because of its picturesque appearance (Houtman 2002). In Japan, the leaves of this shrub are used as vegetables because of their high vitamin C content, and the plant is exploited as a pharmaceutical against *Diabetes mellitus* (Yamada 1998; Tabuchi et al. 2003). *E. gracilistylus* extracts have been reported to inhibit the proliferation of various human tumour cell lines (Shan et al. 2000) and is also commonly applied for the treatment of rheumatic diseases (Yao and Chang 1996). *E. senticosus* is cultivated globally for applications in medicine (Wiersema and León 1999). For example, extracts of rhizomes and roots strengthen the immune system weakened by acute influenza infections (Aicher et al. 2001).

In the past the cultivation of *E. sieboldianus* has been difficult because of the low germination rate and seed dormancy (Rice 1987; Ahn 1993). However, in vitro cultivation techniques have been successfully established for *E. sieboldianus*. Surface sterilized leaf explants are stimulated to form callus on plant nutrient Gelrite medium resulting in the regeneration of numerous somatic embryoids and plantlets after several months. Embryogenic callus

or non-embryogenic callus cultures from the two other closely related species, *E. senticosus* and *E. gracilistylus*, respectively, have been achieved. In vitro cultivation of *Eleutherococcus* plants has been observed to be accompanied by the appearance of microorganisms, predominantly bacteria, which were initially regarded as undesired contaminants. Microorganisms associated with higher organisms can be regarded as natural microflora providing important contributions to developmental processes. To determine if this association is arbitrary or genus-specific three different *Eleutherococcus* species (*E. sieboldianus*, *E. senticosus* and *E. gracilistylus*) were included in our studies which were obtained from three different places.

According to a concept originally made by Lederberg (2004) human beings no longer can be considered as individuals who are genetically defined by only one genome. Cooperative arrangements with numerous microorganisms form a unique microbiome. Similarly, plants living in natural environments represent "superorganisms" (Sapp 2004) or complex micro-ecosystems (Fiore and Gallo 1995) including several endo- and epiphytic microbes. Likewise, plant tissue cultures can harbour endophytic microorganisms. These microorganisms are introduced into in vitro cultures via explants (Leifert et al. 1994; Leifert and Waites 1990, 1994), air or humans (Leifert et al. 1989; Leifert and Cassells 2001). Endophytic bacteria have been reported to live in protoplast cultures of *Discorea* (Mantell 1997, 1998), callus cultures of *Phoenix dactylifera* (Leary et al. 1986), garlic (Fellner et al. 1996), Norway spruce (Ewald et al. 2000), *Pyrethrum* (Bergant et al. 2005), embryogenic cultures of Norway spruce (Ewald et al. 1997), meristem-tip cultures of *Hydrangea* (Cassells and Tahmatsidou 1996), shoot cultures of birch (Gordon and Brown 1988), *Cynara scolymus* (Peñalver et al. 1994), mint (Reed et al. 1995), *Robinia pseudoacacia* (Naujoks et al. 2000), *Echinacea* (Lata et al. 2006), papaya (Thomas et al. 2007a, b) and in leaves and roots of *Zantedeschia aethiopica* plantlets (Gunson 1992) and of *Vitis vinifera* (Thomas and Prakash 2004) under in vitro conditions. Endophytic fungi were found in roots and leaves of micropropagated plants from *Bouteloua eriopoda* and *Atriplex canescens* (Barrow et al. 2004), in shoot tip cultures of pejibaye (de Almeida et al. 2005), apple (Kolozsvári-Nagy et al. 2005) and in suspension cell cultures of *B. eriopoda* (Lucero et al. 2008). Another interesting association with microorganisms was discovered in protoplasts of *Ginkgo biloba* in which unicellular green alga of the genus *Coccomyxa* live inside these cells (Trémouillaux-Guiller et al. 2002). In addition, amoebal species have been isolated from the latex of *Euphorbia marginata* (Troll 1968) and from *Lactuca sativa* (Franchini 1922). Amoebae were also found in the cortex of the roots of *Apium nodiflorum* (Cook 1932) and *Pisum sativum* (Darbyshire and Greaves 1971). These examples show that microbes can form stable associations with plants or plant cells in artificial and natural environments.

Past efforts by others have proven the existence of endophytic fungi in roots of *Aralia elata* and *A. continentalis* (Paul et al. 2007), endophytic bacteria in roots of *Panax ginseng* (Cho et al. 2007) and epiphytic bacteria of *Hedera helix* (Schreiber et al. 2005) grown in natural locations. The aim of this paper was to study the diverse microflora resident in three species of *Eleutherococcus* in vitro cultures. Our hypothesis is that microinhabitants might be present in small numbers and may not be cultivatable. Therefore, a PCR based methodology was selected for analysis of the microinhabitants. Tissue samples are subjected to isothermal amplification of total DNA by the Phi-29 polymerase followed by PCR amplification of rDNA regions using specific primers and cloning of the amplification products to determine the nucleotide sequences of individual amplification products. Thereby, sequences of microorganisms only present in extremely small quantities can be obtained once they have been cloned.

Materials and methods

Plant material

Stem cuttings of *E. sieboldianus* were collected in Beal Botanical Garden, Michigan State University, Lansing, USA. The specimen used in our experiments originated from leaf explants, surface sterilized by 0.15% (w/v) $HgCl_2$ solution for in vitro cultivation. Seeds of *E. gracilistylus* were obtained from Hangzhou Botanical Garden, People's Republic of China, and their germination took place in the Botanical Garden of Marburg in Germany. Surface sterilized leaf explants of *E. gracilistylus* were used for further in vitro experiments. The tissue cultures of *E. senticosus* were generated from embryos in Beijing University of People's Republic of China. The embryogenic calli were induced on nutrient medium GaV (nutrient medium B5) according to Gamborg et al. (1968) supplemented with 200 mg/l spermidine, 1.5 mg/l 2,4-dichlorophenoxy acetic acid, 0.1 mg/l 1-naphtyl acetic acid, 0.1 mg/l indole-3-butyric acid and 0.25 mg/l kinetine. Incubation was at 23 ± 2°C in the dark. The arising embryoids were subcultivated on nutrient medium A3 (basal nutrient medium) according to Linsmaier and Skoog (1965) with 0.15 mg/l 6-benzylaminopurine, 0.03 mg/l kinetine, 0.001 mg/l 1-naphtyl acetic acid, and 0.5 mg/l gibberellic acid. Incubation was at 23 ± 2°C in day light. These cultures were always transferred to new medium in separate operations in order to avoid cross contamination. To induce the differentiation of roots and leaves the embryoids were laid onto nutrient medium A3 devoid of phytohormone substitutions

under sterile conditions, but the embryos were not surface sterilized the same way the leaves were. The incubation period was 3 months when tissue samples were removed for DNA amplification experiments.

Microscopic examination

The plant material was analysed by a Leitz-microscope, model DMRB, by 200× or 1,000× magnification. The microscope was outfitted with a Leica DFC 280 (Leica, Wetzlar, Germany) digital camera. Amoebal cysts were stained according to the method of Phillips and Hayman (1970).

Isolation and cultivation of bacteria

Segments of *E. sieboldianus* in vitro roots were layed on bacteriological medium 20E agar plates (Werner et al. 1975) and incubated at 28°C to grow the bacteria away from the plant roots. Afterwards the isolated bacteria were diluted and streaked on agar plates to obtain isolated colonies.

Amplification and cloning of ribosomal DNA from cultivated bacteria

The bacteria were purified and grown in liquid medium to isolate genomic DNA following the method of Müller et al. (1995). This template was subsequently used to synthesize full length 16*S* rDNA amplification products using the primers fD1 (ccgaattcgtcgacaacAGAGTTTGATCCTGGC TCAG) and rD1 (cccgggatccaagcttAAGGAGGTGATCC AGCC) (Weisburg et al. 1991). The amplification products were ligated to pCR2.1 TOPO vectors (Invitrogen, Carlsbad, California, USA) before determining the nucleotide sequences of purified recombinant plasmids.

Amplification and cloning of ribosomal DNA from non-cultivated bacteria

Seven different samples (Table 1: #31–36, and 41) were separated from callus, somatic embryoids or plantlets of three different *Eleutherococcus* species (*E. sieboldianus*, *E. gracilistylus*, and *E. senticosus*) and were subjected to isothermal genomic DNA amplification, using the GenomiPhi amplification system (GE Healthcare Europe GmbH, Munich, Germany) and following the instructions provided with the kit. The genomic amplification products were subsequently used to specifically amplify the 16*S* rDNA. Polymerase chain reaction was carried out using the Phusion polymerase (BioCat, Heidelberg, Germany) in combination with primers 799f (AACMGGATTAGATACCCKG) and 1492r (ACG GTTACCTTGTTACGAC) (Chelius and Triplett 2001).

Amplification and cloning of ribosomal DNA from non-cultivated fungi

The total DNA samples described in the previous paragraph were used as templates for PCR-amplification of eukaryotic rDNA regions. Internal transcribed spacer regions ITS1 and ITS2, including the 5.8*S* rDNA subunit were PCR-amplified in combination with primers ITS4 (TCCTCCGCTTATTG ATATGC) and ITS5 (GGAAGTAAAAGTCGT-AACAA GG) (White et al. 1990). Primers EF4 (GGAAGGGRTGTA TTTATTAG) and EF3R (TCCTCTAAATGACCAGTTTG) (Smit et al. 1999) and primers NS3 (GCAAGTCTGGTGCC AGCAGCC) and NS8 (TCCGCAGGTTCACCTACGGA) (White et al. 1990) were applied to obtain 18*S* rDNA amplification products. Primers ITS2 (CGCTGCGTTCTT CATCG), 5.8*S* 37-40 (CATCGTTACAAGAGCCAAG) or 5.8*S* 34-40 (CCGCGCTCTTCATCG) were combined with primer EF4 to obtain sequences overlapping the SSU and ITS1 region.

Isolation of microorganisms by a three-dimensional micromanipulator

Gelrite medium plates with *E. sieboldianus* in vitro cultures were examined for the presence of altered root hairs, infected by microorganisms, or screened for the occurrence of pale halos, indicating microbial growth in the rhizosphere. Sterilized pipette tips or glass micropipettes were used to collect samples. A dilution series of the sample material was streaked on a surface sterilized glass plate. Individual microbial cells or small groups of cells were collected by a microcapillary pipette, which was mounted to a three-dimensional (3D) micromanipulator (Institut für Mikrobiologie und Weinforschung, Johann Gutenberg Universität, Mainz). The cells were transferred into sterilized microcentrifuge tubes and stored on ice. Subsequent isothermal DNA amplification was carried out within a few hours after isolation of cells.

DNA sequencing

Prior to DNA sequencing, PCR amplification products were ligated to pCR2.1-TOPO or pCRblunt-TOPO vectors (Invitrogen Corp., Carlsbad, California, USA). DNA sequencing was carried out as described elsewhere (Becker et al. 1998) using the DNA-sequencing kit by Fermentas in combination with IRD800-labelled primers (24mers, reverse 49 and forward 40) synthesized by MWG Biotech, Eberswald, Germany, and a LI-COR sequencing machine (LI-COR Biosciences GmbH, Bad Homburg, Germany). Alternatively, sequencing was done by Genomex, Amplicon Express, Pullman, Washington USA, or by MWG Biotech, Eberswald, Germany. Sequence alignments were carried out by using the

Table 1 Identification of microorganisms by sequence comparison of rDNA and ITS regions

Tissue	Primers	Sequences identified (data base references)	
E. sieboldianus			
Exudates	fD1–rD1	*Stenotrophomonas*	Ib-14 (EU816934)
		Paenibacillus	L-9 (EU816937)
			W-4 (EU816939)
		Pseudomonas	G-2-2 (EU816940)
	799f–1492r	*Microbacterium*	r-43 (EU816944)
		Stenotrophomonas	l-43 (EU816933)
			Ib-14 (EU816934)
			Ib-16 (EU816935)
			l-35-2 (EU816936)
		Paenibacillus	L-9 (EU816937)
			x47-2 (EU816938)
			W-4 (EU816939)
		Pseudomonas	G-2-2 (EU816940)
			y49-2 (EU816941)
		Unidentified bacterium	p42 (EU816943)
Single cell isolated from rhizosphere	EF4–EF3R	Uncultured Basidiomycete	MM-5 (EU812508)
Group of coccoid cells isolated from rhizosphere	799f–1492r	Uncultured bacterium	MM9-4 (EU816942)
Group of cells isolated from root hair	EF4–EF3R	Uncultured Basidiomycete	MM-13 (EU812509)
31 Root apex	799f–1492r	*Paenibacillus*	MDM31 (EU816949)
	ITS4–ITS5	*Eleutherococcus* (chloroplast)	
	NS3–NS8	*Mesorhizobium*-like	W31-5 (EU816928)
32 Subapical root		*Paenibacillus*	MDM32c (EU816947)
33 Leaf	ITS4–ITS5	*Eleutherococcus* (chloroplast)	
34 Brownish callus	799f–1492r	*Paenibacillus*	MDM34 (EU816948)
	ITS4–ITS5	*Eleutherococcus*	
		Mallomonas-like	34-1 (EU812485)
	EF4–EF3R	Uncultured fungus	34-47 (EU812494)
			34-13 (EU812495)
			34-13 (EU812496)
			34-47 (EU812497)
			34-1 (EU812498)
			34-34 (EU812505)
			34-42 (EU812504)
	NS3–NS8	Uncultured α-proteobacteria	W34-2 (EU816929)
35 Brownish callus	799f–1492r	*Paenibacillus*	
	ITS4–ITS5	*Eleutherococcus*	
		Malassezia-like	35-1 (EU812484)
		Uncultured fungus	35-3 (EU812499)
			35-11 (EU812500)
			35-12 (EU812501)
	NS3–NS8	Uncultured α-proteobacteria	W35-4 (EU816932)
36 Shoot	799f–1492r	*Paenibacillus*	MD36 (EU816945)
	EF4–EF3R	Uncultured fungus	36-13 (EU812502)
			36-15 (EU812506)
			36-24 (EU812503)
			36-28 (EU812507)
	NS3–NS8	Uncultured *Pseudomonas*	W36-3 (EU816931)

Table 1 continued

Tissue	Primers	Sequences identified (data base references)	
41 Embryogenic callus	799f–1492r	Uncultured bacterium	MDM41k (EU816946)
	NS3–NS8	Uncultured *Pseudomonas*	W41-5 (EU816930)
E. gracilistylus			
38 Non-embryogenic callus	ITS4–ITS5	*Geomyces*	38-15 (EU812475)
		Vahlkampfia	38-21 (EU812477)
			38-2 (EU812478)
		Uncultured fungus	38-7 (EU812479)
			38-22 (EU812476)
		Mallomonas-like	38-1 (EU812480)
E. senticosus			
40 Non-embryogenic callus	ITS4–ITS5	*Mallomonas*-like	40-4 (EU812486)
		Unidentified organism	40-6 (EU812487)
			40-2 (EU812488)
			40-3 (EU812489)
			40-15 (EU812492)
		Vahlkampfia	40-1 (EU812490)
			40-11 (EU812491)
			40-16 (EU812493)

Accession numbers of sequences obtained are presented in brackets

DS Gene 1.5 programme (Accelrys, Cambridge, UK). DNA sequencing experiments were carried out at least with threefold replications to obtain reproducible and reliable data. DNA sequences were compared with the NCBI data base (nblast). Nucleotide sequences were compared by multiple alignments, using clustalW and ARB (Ludwig et al. 2004).

Results

Microorganisms observed in the rhizosphere and in root hairs

Detailed examination of in vitro cultures revealed manifold structural abnormalities of root hairs, which were branched, curled, swollen or screwed (Fig. 1a–d). Microorganisms, individually and in colonies were observed within the rhizosphere. Coccoid (size: 2–3 μm) and rod-shaped (size: upto 10 μm) microorganisms were detected within root hairs (Fig. 1e–g), moving actively towards the root hair tip and back again, apparently driven by flagella (Fig. 1e). Occasionally, root hair tips were capped by densely packed colonies of micro-organisms (Fig. 1h).

Isolation and identification of cultivated bacteria

Laying surface sterilized root segments on agar medium 20E and subsequent incubation at 28°C over night resulted in the formation of different bacterial colony types. One colony type was smooth and coloured intensely yellow, another colony type was also yellow, but the colonies had an irregular shape, the surface was rough and wrinkled. A third colony type looked pale and smooth. DNA sequencing revealed that the closest relatives were found among the family of Pseudomonadaceae, *Paenibacillus* sp. and *Stenotrophomonas* sp. (Table 1, exudates).

16*S* rDNA analysis of non-cultured bacteria

In addition to rhizobacterial isolates, eleven different tissue samples were taken from callus, somatic embryoids and plantlets of three different *Eleutherococcus* species, which then were subjected to isothermal DNA amplification. Samples are numbered from 31 to 41 (see Table 1) The genomic amplification products were used for 16*S* rDNA amplification. For this purpose a pair of specific primers (799f and 1492r) was applied to avoid the amplification of *Eleutherococcus* plastid-derived products. Apart from genera already identified by the analysis of cultivated isolates, this approach led to the identification of several new sequences, related to *Pseudomonas*, *Mesorhizobium* and other α-proteobacteria, as well as to uncultured bacteria. *Paenibacillus* was obtained from brownish embryogenic callus, root and shoot of *E. sieboldianus* (Table 1).

Fungal and protozoan ITS regions identified in *Eleutherococcus* sp. in vitro tissue samples

The identical DNA isothermal amplification products obtained from the tissue samples described in the previous

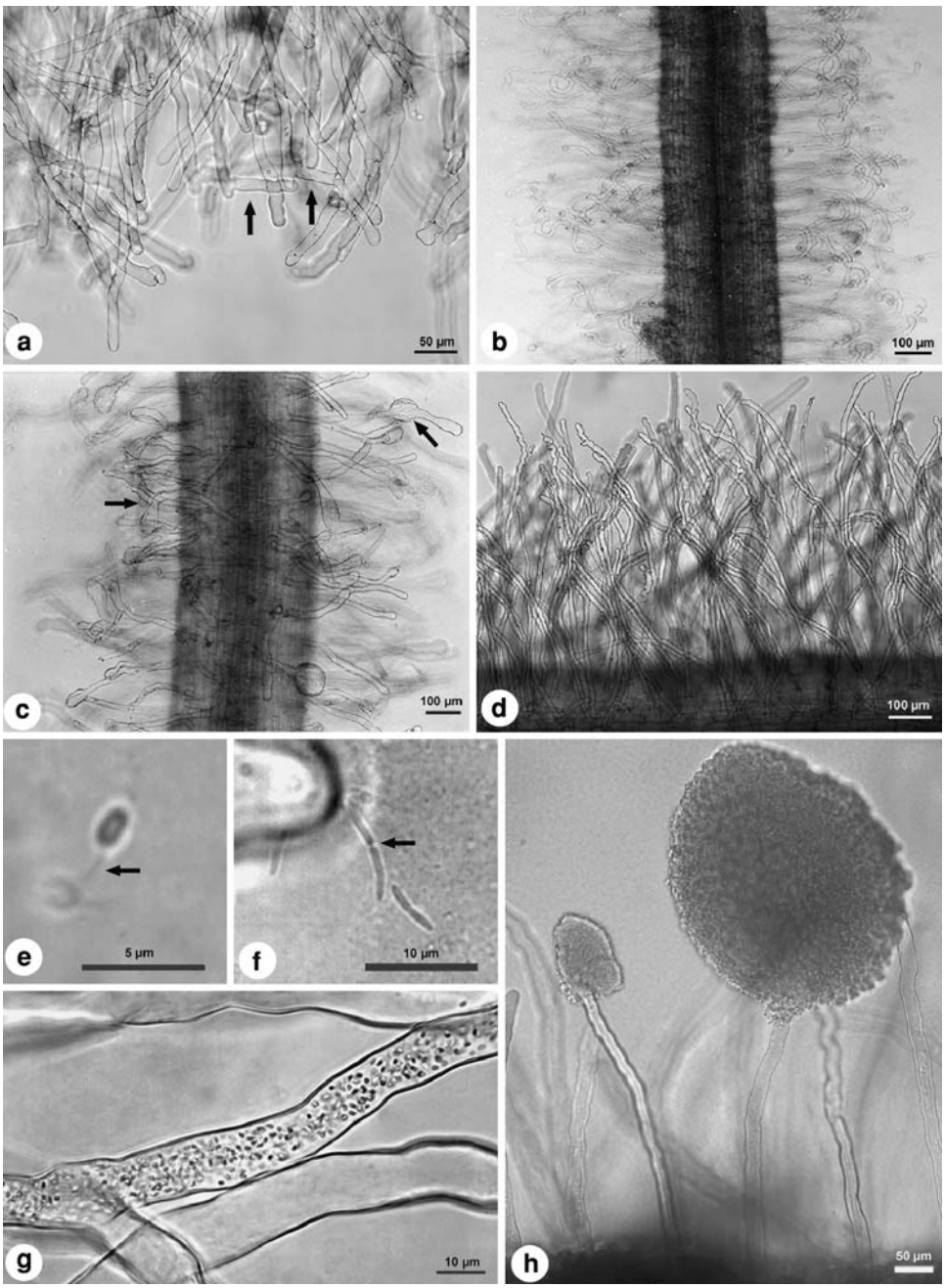

Fig. 1 Microbes living in *E. sieboldianus* root hairs regenerated from plant tissue cultures. Deformed root hairs. **a** Branched (*arrow*), **b** curled, **c** swollen (*arrow*) and **d** screwed root hairs. Microbes isolated from root hairs. **e** Oval microbe with flagellum (*arrow*). **f** Rod-like microorganisms, prior to cell division (*arrow*). **g** Root hair filled with oval microbes, and non-colonized root hairs. **h** Root hairs densely packed with microorganisms, forming a continuum with apical caps

section were used to multiply the complete ITS1 and ITS2 regions (including the 5.8*S* rDNA) of fungal species. Comparison of DNA sequences with sequences in the NCBI data base indicated at least eight different groups of eukaryotic microorganisms (Table 1). One group, strongly similar to *Mallomonas* (a flagellate), was found in four different tissue samples representing three different plant species (#34, brownish callus of *E. sieboldianus*; #37, leaf of *E. sieboldianus*; #38, non-embryogenic callus of *E. gracilistylus*; #40, non-embryogenic callus of *E. senticosus*). Another series of sequences, with similarity to *Vahlkampfia* was detected in callus of *E. gracilistylus* and *E. senticosus*. *Vahlkampfia* is a free-living amoeba found in soil (Rodriguez-Zaragoza and Garcia 1997; Garstecki et al. 2005; Rodriguez-Zaragoza et al. 2005). Additional DNA sequences, exhibiting sequence similarities to uncultured fungi were identified in tissues of *E. sieboldianus* (#35, brownish callus) and *E. gracilistylus* (#38, non-embryogenic callus).

Another DNA sequence revealed an ITS region closely related to *Malassezia* in brownish callus of *E. sieboldianus* (sample #35, Table 1). In addition, ITS sequences similar to *Geomyces* were identified in *E. gracilistylus*, non-embryogenic callus (sample #38, Table 1). Sample #38 is the sample

from which the highest number of different microorganisms has been detected. Several ITS sequences were obtained which could not be classified but were similar to sequences in the database that were related to different groups of organisms, such as fungi, bacteria and protozoa. This limitation is probably due to the fact that corresponding ITS sequences are not yet available in the database.

Light microscopic documentation of amoebae in *E. sieboldianus* roots

rDNA sequence similarities to *Acanthamoeba* and to *Vahlkampfia* were unexpected. Therefore we re-examined the photographs that were taken from the in vitro cultures prior to isolation of tissue samples. Closer inspection, as shown in Fig. 2a revealed amoebae, characterized by numerous pseudopodia, in association with *E. sieboldianus* roots. *Acanthamoeba* and *Vahlkampfia* are known to form cysts which are ellipsoid or irregularly shaped. Examination of micrographs revealed the presence of numerous cyst-like structures. At least three different types were found which were around 10 µm in diameter (Fig. 2c). Occasionally the cysts were found densely packed within an *E. sieboldianus* root cell. This observation supports the conclusion that amoeba were not only associated with *E. sieboldianus* in vitro cultures, but they were living intracellularly in host plant cells (Fig. 2b). Using Trypan-blue as a staining dye, it became evident that one particular type of cysts consisted of a double-layered coat with ornamental decorations on the outer surface (Fig. 2c).

Fungal 18*S* rDNA regions identified in *Eleutherococcus* sp. in vitro tissue samples

When primers EF4 and EF3R were used in specific PCR amplification experiments, products were obtained from templates #34 (brownish callus) and #36 (shoot). DNA sequence analysis confirmed that the amplification products encoded 18*S* rDNA of non-cultivated fungi and revealed almost perfect identity with an uncultured fungus partial 18*S* rRNA gene, clone WIM108, identified in a soil sample from top 0 to 25 cm layer of non-fertilized agricultural field, collected in 1975 (AM114819). Unfortunately, PCR primers EF3R and ITS5 bind to adjacent rDNA regions and do not lead to overlapping amplification products. Therefore, partial sequences obtained from 18*S* rDNA and ITS regions, could not be combined without uncertainty.

Molecular characterisation of microorganisms isolated by a 3D micromanipulator

The high number of bacterial and fungal species identified in tissue samples of surface sterilized *Eleutherococcus* sp. in vitro cultures raised the question whether the microorganisms had in fact been isolated from the plant cells or whether they originated from the tissue or were caused by sample contamination. Therefore, a new series of *E. sieboldianus* tissue cultures were grown. Microscopic analysis was carried out in a "neutral" laboratory, using a 3D micromanipulator. Again, plant root hairs filled with microorganisms were

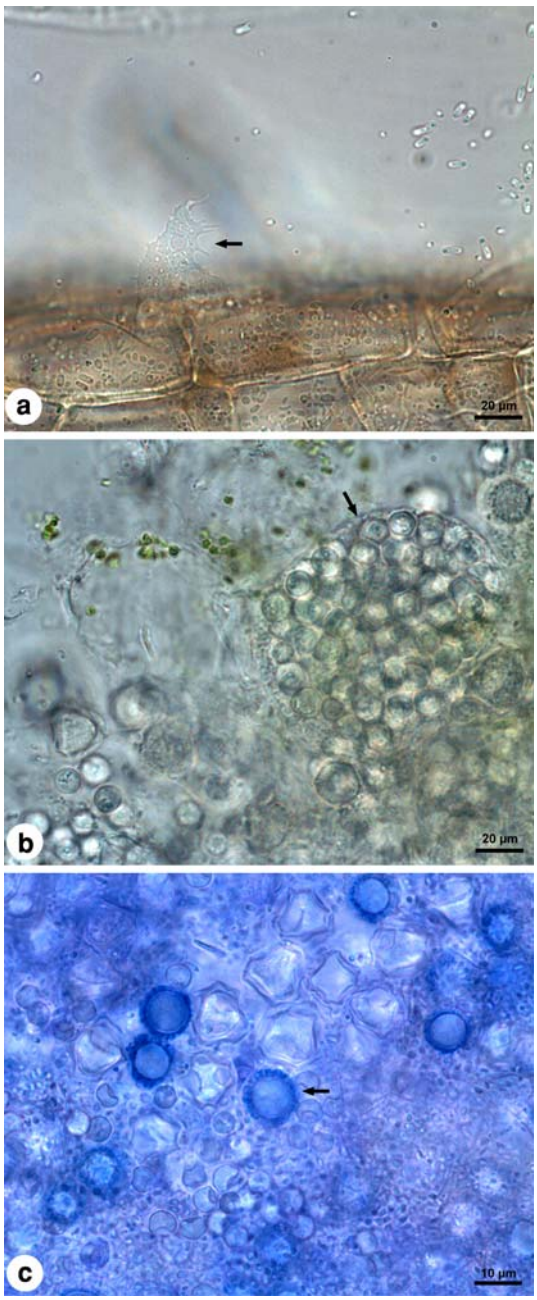

Fig. 2 Amoebae associated with *Eleutherococcus* in vitro cultures. **a** amoeboid cell (*arrow*) living on the root surface of *E. sieboldianus*, **b** *E. sieboldianus* in vitro root cell (*arrow*), densely filled with amoebal cysts, **c** several isolated *E. sieboldianus* cells containing double-layered amoebal cysts (*arrow*) with decorations on the outer surface, made visible by higher magnification and Trypan-blue staining

observed which subsequently were isolated from their hosts. The isolated cells then were subjected to an isothermal DNA amplification, providing sufficient genomic DNA for specific DNA amplification experiments.

Using primers 799f and 1492r (specific for the 3′ end of prokaryotic 16*S* rDNA regions) resulted in the identification of sequences related to uncultured bacteria. Using primers EF4 and EF3R (specific for fungal 18*S* rDNA regions), high sequence similarities were obtained to non-cultivated fungi, especially to the uncultured basidiomycete partial 18*S* rRNA gene of a single cell isolated from the rhizosphere, and from a group of cells isolated from a root hair.

Discussion

Initial investigations with *E. sieboldianus* plant tissue cultures revealed the presence of microorganisms in the rhizosphere and within the root hairs, although the plantlets had been regenerated from surface-sterilized leaf tissue. The root hair deformations of *E. sieboldianus* in vitro plantlets resemble those observed with other microbes like *Rhizobium leguminosarum* bv *viciae* in *Vicia sativa* (Heidstra et al. 1994), *Rhizobium meliloti* in *Medicago truncatula* (Ardourel et al. 1994), *Rhizobium trifolii* in *Trifolium repens* (Ervin and Hubbell 1985), *Azospirillum brasiliense* in *Fragaria* × *ananassa* (Bellone and de Bellone1995) and *Triticum aestivum* (Fedonenko et al. 2001) and *Ligniera pilorum* in *T. aestivum* (Barr 1979). However, this does not necessarily mean similar modes of infection in the Fabales/*Rhizobium* symbiosis compared to the *Eleutherococcus* in vitro systems, and the causative interrelationship between *Eleutherococcus* sp. and its different microinhabitants remains to be determined.

A number of bacteria were cultivated on agar medium, indicating that these were not obligate biotrophic microbes. While it was relatively easy to cultivate rhizosphere bacteria on agar medium which then could be used for further analyses, it was an experimental challenge to gain sufficient sample material of the intracellular microbes. Starting from very small tissue samples (<1 mm^3), genomic DNA was enriched by isothermal amplification based on polymerase Phi-29, resulting in high DNA concentrations. To our knowledge, in this study polymerase Phi-29 has been used for the first time to amplify DNA of microorganisms within plant tissues. The choice of primers used for specific DNA amplification of bacterial 16*S* rDNA and the subsequent ligation to pCR2.1-TOPO vectors led to the separation of individual amplification products and thus to the identification of numerous bacterial species belonging to taxonomically diverse groups related to Firmicutes (*Paenibacillus* sp.) and γ-proteobacteria (*Pseudomonas* and *Stenotrophomonas*). The fact that identical species were detected in different tissue samples indicates that these bacteria are systemically distributed in the plants. This is not only restricted to differentiated plant tissue such as root apex, shoot and leaf, because a considerable number of different bacterial species were detected in samples taken from embryogenic callus. Therefore we have to conclude that regenerating plants are infected by these bacteria from the beginning. Close relatives of some of these non-cultured bacteria have been reported in association with plants grown in Asia, such as *E. sieboldianus*, and to have plant growth promoting traits (Chung et al. 2000; von der Weid et al. 2003). This observation suggests that the intracellular bacteria have been sustained for very long through the evolutionary development and that the occurrence of these bacteria should not be regarded as an artificial contamination. It appears likely that this plant species is not an exception, but that plants in general are settled by bacteria (Hallmann et al. 1997) and fungi (Petrini 1986; Carroll 1988). As an example, *Paenibacillus* was identified in tissue cultures of woody plants as a predominant endophytic bacterium (Ulrich et al. 2008).

Regarding the distribution of the identified bacteria there are also places in other plant tissue cultures. Species of *Stenotrophomonas* were also found in in vitro shoots of *Echinacea pallida* var. *angustifolia* (Lata et al. 2006), in *Dieffenbachia* sp. cultures (Jan et al. 2004) and in in vitro leaves and flower stalks of *Limonium sinuatum* (Liu et al. 2005). Species of *Stenotrophomonas* like *Stenotrophomonas maltophilia* (Hauben et al. 1999) are also distributed by man. Thus it could be that these bacteria were transferred from laboratory staff members in in vitro culture of *Eleutherococcus*. Otherwise *S. maltophilia* lives as an endophyte in cotton and sweet corn plants (McInroy and Kloepper 1995). The *Eleutherococcus* plants could naturally harbour this bacterial species.

The size of flagellated microorganisms (10 μm) observed by light-microscopic analysis suggested the presence of eukaryotic microbial species within the plant root hairs. Therefore the genomic DNA amplification products were also examined by a pair of primers suitable for amplifying fungal ITS regions. White et al. (1990) introduced primers ITS4 and ITS5 for the taxonomic characterization of fungal species. In the course of our experimental studies these "fungal primers" turned out to have a fairly low specificity such that, besides fungal DNA, we amplified rDNA from several protozoan genera (*Vahlkampfia*, *Vanella* and *Mallomonas*) and also from the host plant *Eleutherococcus*. Similar observations were made by Johnston et al. (2005) who used this primer pair for molecular phylogenetic analyses of different Brassicaceae species. The fungal primers NS3 and NS8 designed by White et al. (1990) also had a low specificity. In our analyses, the amplification products

obtained were related to bacterial DNA. The resulting sequence data were surprising because the number of different fragments was higher and taxonomically more diverse than expected.

The flagellated coccoid microbes could be flagellated zoospores taxonomically related to Chytridiomycota. For example, *Johnkarlingia brassicae* develops very small zoospores which are 0.5–1 μm in size (Singh and Pavgi 1979). The length of *Synchytrium trichosanthidis* zoospores is 2–4 μm (Raghavendra and Pavgi 1979).

Furthermore it is intriguing that several other fungi were detected particularly in callus of two *Eleutherococcus* species. These are related to *Malassezia* and *Geomyces* among several additional less well-characterized species in the NCBI data base. Since ITS regions are highly diverse, 18*S* rDNA sequences were established to obtain more precise ideas about the taxonomy of these fungal species. These results confirm that the microorganisms are related to the fungal species as already indicated by the ITS sequence comparisons. The fungus *Malassezia* is a microorganism living on animal and human skin (Aspiroz et al. 1999; Chen and Hill 2005) and is associated with soil nematodes (Renker et al. 2003). The *Eleutherococcus* plants growing in botanical gardens and used for establishing in vitro cultures could be infected with *Malassezia* fungi by humans.

Using a 3D micromanipulator to isolate and separate individual bacterial and fungal cells from living *Eleutherococcus* root hair cells has proven to be a powerful tool for analysing the intimate relationship between the host plant and its microinhabitants. By this means it was possible to isolate single cells or small groups of cells and to specifically amplify genomic DNA.

In the very small tissue samples we did not find any fungal hyphae. Otherwise we identified DNA of several fungal species that are known to form hyphae. This contradiction might be explained by the possibility that we are dealing with dimorphic fungi developing a mycelium as well as single fungal cells (yeast-like cells). Most likely it is extremely difficult to detect single fungal cells by light microscopy, due to their low number and small size. The fungal genus *Malassezia* identified in our studies, are known to be dimorphic fungi (Kreisel and Schauer 1989). Another explanation could be that the fungi identified as uncultured fungi, and *Geomyces* produce mycosomes under in vitro conditions. The mycosomes are round minute fungal propagules which are distributed in cells of various plant species (Atsatt 2003).

The fact that amoebal cysts were detected in root cortex cells of in vitro propagated *E. sieboldianus* plants and the finding that amoebal rDNA sequences were identified in samples isolated from non-embryogenic callus structures of *E. gracilistylus*, favours the assumption that amoebae are commonly present in *Eleutherococcus* sp. cells. The translucent appearance of amoeboid cells could be another reason why these microinhabitants of plant tissues and inside plant cells have been overlooked in the past. It was the combined approach by microscopic observation and comparative sequence analysis which lead to the identification of amoeba in *E. sieboldianus* in vitro cultures. Since the in vitro cultures have been grown with particular care and under strictly sterile conditions, a contamination with amoeba by air or by contaminated nutrient solutions can be considered unlikely.

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