Characterization and utilization of bacterial endophytes during in vitro culture of wild cherry (*Prunus avium* L.)

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktorin der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

Dipl.-Biol. Mona Quambusch

2016

Referentin:	Prof. Dr. rer. hort. Traud Winkelmann
Korreferenten:	Prof. Dr. rer. nat. Thomas Debener, Prof. Dr. rer. nat. Kornelia Smalla
Tag der Promotion:	06. Juli 2016

„Honour thy symbionts“

Jian Xu and Jeffrey I. Gordon

Abstract

Wild cherry (*Prunus avium* L.) is a hardwood cultivated for the production of high quality furniture. To optimize growth characteristics (fast growth, straight stem) single plants showing a favourable habitus have been selected and propagated as in vitro clones under the trademark silvaSELECT®. During in vitro propagation high scale losses of plantlets have been observed, especially during rooting and acclimatization. It was supposed, that these are caused by the presence or absence of endophytes in micropropagated plantlets. Endophytic bacteria occurring in plant in vitro cultures have often been described as contaminations, although they are generally present in all pant tissues, often with plant growth promoting effects. This thesis focuses on the positive effects endophytes can have on plants and aims to characterize and utilize the bacteria to improve in vitro culture propagation of *P. avium*.

The endophytic bacterial communities of *P. avium* genotypes differing in their growth patterns during in vitro propagation were analyzed. In a culture-dependent approach five morphologically distinct isolates from tissue culture material were identified by 16S rDNA and ITS sequences. To analyze the uncultivable fraction of the bacteria, a clone library was established from amplified 16S rDNA of total plant extract and analyzed by amplified ribosomal DNA restriction analysis (ARDRA) and sequencing of one clone for each identified OTU. While all *Prunus* genotypes contained *Mycobacterium* spp., two other bacterial genera, *Rhodopseudomonas* spp. and *Microbacterium* spp., were detected in high abundance only in two easy-to-propagate genotypes. Thus, the community structures differed significantly between cherry genotypes and bacterial genera with previously reported plant growth-promoting effects were only detected in genotypes with high propagation success.

These data indicated a possible positive impact of the bacteria on in vitro propagation of *P. avium*. The two isolates *Rhodopseudomonas palustris* N-I-2 and *Microbacterium testaceum* D-I-1 were therefore tested in an inoculation experiment, and showed a promoting effect on rooting of two difficult-to-propagate genotypes. Both bacterial species led to higher rooting percentages and a significantly higher root number of both *P. avium* genotypes.

Genus specific quantitative PCR protocols were established to look at the dynamics of the most abundant endophytes, *Mycobacterium* spp., *Rhodopseudomonas* spp. and *Microbacterium* spp. The bacterial DNA amount was quantified during propagation and rooting in the different plant organs, and in three successive years of propagation. All three endophyte groups were detected in stem, leaf and root tissue of both culture phases and a high fluctuation in the abundance between sampling times was observed. In addition, it was shown that after regeneration via adventitious shoots the bacteria were not eliminated, but showed slightly modified concentrations.

Next to the analysis of the endophytic community an experiment to optimize the in vitro rooting phase as the major limiting factor of *P. avium* micropropagation was conducted. The influence of a four days dark treatment and a 48 h IBA pulse on in vitro root formation of *P. avium* microshoots was examined. Both treatments led to significantly higher rooting percentages in the majority of the six analyzed genotypes and thereby contributed to an effective production protocol during commercial production of *P. avium*.

Keywords: *Prunus avium*, in vitro propagation, endophytes

Zusammenfassung

Die Vogelkirsche (*Prunus avium* L.) gehört zu den Edellaubhölzern und wird in Deutschland vor allem zur Verwendung in der Möbelproduktion angebaut. Um Bäume mit den gewünschten Wachstumseigenschaften (geradschaftiges und schnelles Wachstum) zu selektieren wurden einzelne Pflanzen mit geeignetem Habitus ausgewählt, als In-vitro-Klone vermehrt und werden unter dem Markenzeichen silvaSELECT® vertrieben. Während der Vermehrung treten hohe Verluste durch eine geringe Bewurzelungsrate der Mikrostecklinge und niedrigen Akklimatisierungserfolg auf. Es wird angenommen, dass diese auf das Vorhandensein oder Fehlen von Endophyten zurückzuführen sind. Endophytische Bakterien in In-vitro-Kulturen wurden bisher meist als Kontaminationen wahrgenommen, die die Vermehrung negativ beeinflussen. Sie kommen jedoch generell in allen Pflanzengeweben vor und werden oft mit pflanzenwachstumsfördernden Eigenschaften in Verbindung gebracht. In dieser Arbeit sollten die positiven Eigenschaften von Endophyten auf die Pflanze im Vordergrund stehen, die Bakterienpopulation charakterisiert und die gewonnenen Informationen für eine Verbesserung der In-vitro-Vermehrung von *P. avium* genutzt werden.

In einem kulturunabhängigen Ansatz wurden fünf morphologisch klar unterscheidbare Bakterienstämme isoliert und anhand der 16S rDNA und ITS Sequenz identifiziert. Zur Analyse der unkultivierbaren Bakterienfraktion wurde eine Klonbibliothek aus amplifizierter 16S rDNA direkt aus dem Pflanzenmaterial erstellt und mittels ARDRA (amplified ribosomal DNA restriction analysis) und Sequenzierung eines Klons für jede detektierte OTU analysiert. Die Gattung *Mycobacterium* konnte in allen vier untersuchten Kirschgenotypen nachgewiesen werden. In gut vermehrbaren Genotypen wurden zusätzlich *Rhodopseudomonas* spp. und *Microbacterium* spp. detektiert. Die Bakterienstruktur unterschied sich damit wesentlich zwischen den Genotypen; Bakterien mit bereits in der Literatur beschriebenen wachstumfördernden Eigenschaften wurden aussschließlich in gut vermehrbaren Genotypen gefunden.

Mit den potentiell wachstumsfördernden Isolaten *Rhodopseudomonas palustris* N-I-2 und *Microbacterium testaceum* D-I-1 wurde ein Inokulationsexperiment durchgeführt. Für beide Bakterien konnte eine bewurzelungsfördernde Wirkung bei zwei schwer vermehrbaren Genotypen in Form eines höheren prozentualen Anteils an bewurzelten Pflanzen und einer höheren Anzahl Wurzeln pro Pflanze nachgewiesen werden.

Zur Quantifizierung der Bakterien im Pflanzenmaterial wurden spezifische PCR-Primer entwickelt. In sechs *Prunus avium*-Genotypen wurde die Verteilung von *Mycobacterium* spp., *Rhodopseudomonas* spp. und *Microbacterium* spp. in verschiedenen Pflanzenorganen während der Vermehrungs- und Bewurzelungsphase untersucht. Die Veränderung der Bakterienpopulation wurde über drei Jahre beobachtet. Alle drei Bakteriengattungen wurden in allen Organen und in beiden Kulturphasen nachgewiesen. Auffällig war eine starke Fluktuation zwischen den einzelnen Messungen. Zusätzlich wurde gezeigt, dass die Bakterien durch die Regeneration über Adventivsprosse nicht eliminiert wurden, jedoch in ihrer Konzentration leicht verändert vorlagen.

Neben dem Endophytenschwerpunkt wurden Experimente zur Verbesserung der Bewurzelungsphase mit Hilfe einer Dunkelbehandlung (4 Tage) und eines 48 h IBA-Pulses durchgeführt. Beide Behandlungen führten zu signifikant höheren Bewurzelungsraten in der Mehrheit der sechs analysierten Genotypen und trugen damit zu einem effektiveren Produktionsprotokoll während der kommerziellen Produktion von *P. avium* bei.

Schlagwörter: *Prunus avium*, In-vitro-Vermehrung, Endophyten

Contents

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
ANOVA	analysis of variance
ARDRA	amplified ribosomal DNA restriction analysis
BAP	benzylaminopurine
CFU	colony forming unit
DGGE	denaturing gradient gel electrophoresis
dNTP	deoxynucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
GA3	gibberellic acid-3
gDNA	genomic DNA
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IFP	Institut für Pflanzenkultur
ISR	induced systemic resistance
ITS	16S-23S internal transcribed spacer
MAMP	microbe associated molecular pattern
MS medium	Murashige and Skoog medium (1962)
NAA	1-naphthaleneacetic acid
NGS	next generation sequencing
NTC	no template control
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PGPB	plant growth promoting bacteria

PGR	plant growth regulator
qPCR	quantitative real-time polymerase chain reaction
rDNA	ribosomal DNA
rRNA	ribosomal RNA
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SSCP	single strand conformation polymorphism
TDZ	thidiazuron
TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism
VOC	volatile organic compound
WPM	woody plant medium

1 Introduction

1.1 *Prunus avium* – Wild cherry

1.1.1 Introduction of Prunus avium *for its use in forestry*

Wild cherry (*Prunus avium* L.) belongs to the Rosaceae and is the most important European timber species within the family (Russell 2003). Its natural distribution is centered in Europe with extension to western Eurasia and the northern part of Africa within a latitude of 30° to 61° N (Schmid 2006). *Prunus avium* has small, edible, red to black fruits with 1 cm diameter that are disseminated by birds. Although wild cherry is not an endangered species, the genetic diversity of populations can be considered to be under threat due to its generally scattered occurrence and the introgression of widespread domesticated sweet cherry (Russell 2003; Janßen et al. 2010).

The timber of *Prunus avium* is highly valued in Europe due to its reddish color, comparable to tropical trees like mahogany, and the firmness of the wood, combined with fast and fine grained growth (Kobliha 2002; Russell 2003). It is used for the production of solid wood furniture and as veneer in interior fittings (Ducci and Santi 1997; Schmid 2006). Several intensive wood production plantations are being carried out in Europe using wild cherry as a valuable species that produces high quality timber within 60 to 80 years (Ducci and Santi 1997). In addition, it is increasingly used for the reafforestation of marginal agricultural land since the 1990s (Santi et al. 1998; Díaz et al. 2007) and is valued for wildlife and amenity plantings (Turok et al. 1997).

Many European countries have gene conservation and/or breeding programs for wild cherry, e. g. the Czech Republic (Kobliha 2002) and Spain (Fernandez-Cruz et al. 2014). In Germany a program for the vegetative propagation of selected plus trees started in 1984. Trees with optimized growth characteristics, such as fast growth, straight stem and fine grains (see Fig. 1 E for an example) have been selected and are distributed under the trademark silvaSELECT® since 2004 (Meier-Dinkel et al. 2007; Janßen et al. 2010). The clone collection consisted of 28 genotypes[1] and new genotypes were added over the following years to ensure genetic diversity of the afforestation sites as prescribed by the German act on forest reproductive material (Forstvermehrungsgutgesetz, FoVG).

[1] The clones of the silvaSELECT® collection are from here on named genotypes instead of clones to differentiate clearly from bacterial clone bench.

1.1.2 *Propagation of* Prunus avium *by in vitro culture*

Generative propagation of *Prunus avium* is possible, however, the seeds have to go through a cold stratification of five months and the offspring consists of individuals with different growth performances. Both problems are obviated by vegetative propagation, enabling stable clonal propagation of selected plus trees with the desired quality characteristics (Meier-Dinkel et al. 2007). *Prunus avium* selections, e. g. for the use as rootstocks of sweet cherry cultivars, have traditionally been propagated by shoot cuttings. While there are reports of the successful use of root and shoot cuttings (Ghani and Cahalan 1991), *Prunus avium* frequently shows low rooting percentages and the cloning becomes progressively more difficult as the donor trees mature (Al Barazi and Schwabe 1985; Hammatt and Grant 1997). Dick and Leakey (2006) observed 77 % rooting of juvenile softwood cuttings compared to 7 % rooting in softwood cuttings from a 20 year old tree. Micropropagation can lead to a successful rejuvenation and higher rooting efficiency of plants (Hammatt and Grant 1993; Xiao et al. 2014).

Fig. 1 *P. avium* plants during the propagation process (A) Microshoots on rooting medium, genotype Neptun; (B) Two rooted and one unrooted microshoot, genotype Achilleus (C); *P. avium* genotype Neptun plants four weeks and (D) eight weeks after transfer to soil; (E) 25 year old offspring of a selected plus tree (genotype Fama) of the silvaSELECT® clone collection.

One of the first protocols for in vitro propagation of wild cherry, in this case from one year old cherry plants, was published by Jones and Hopgood (1979) and the technique was few years later successfully applied to adult trees, e. g. by Riffaud et al. (1981). Micropropagation proved to be the most effective means to rapidly produce clonal material from adult trees (Hammatt and Grant 1997). Meier-Dinkel (1986) published a

protocol for the micropropagation of selected plus trees with high multiplication rates using five steps: culture establishment, formation of axillary shoots, shoot elongation, rooting, and acclimatization. Since then, the propagation protocol was optimized and simplified to enable the use in commercial practice. The protocol used in this thesis and by the cooperating company of this project is composed of four main culture steps (see Fig. 2):

(1) Establishment of an in vitro culture from winter buds harvested from adult trees. The winter buds are surface sterilized and the outer scale leafs removed before the transfer onto culture medium containing BAP (benzylaminopurine), IBA (indole-3-butyric acid), and GA3 (gibberellic acid-3) to induce elongation and the formation of axillary shoots.

(2) Propagation of microshoots using the same culture medium. Axillary shoots are subcultured every five weeks until the required number of plants is obtained.

(3) Rooting on medium containing IBA to induce the formation of roots.

(4) Acclimatization to soil and greenhouse conditions with gradual reduction of air humidity.

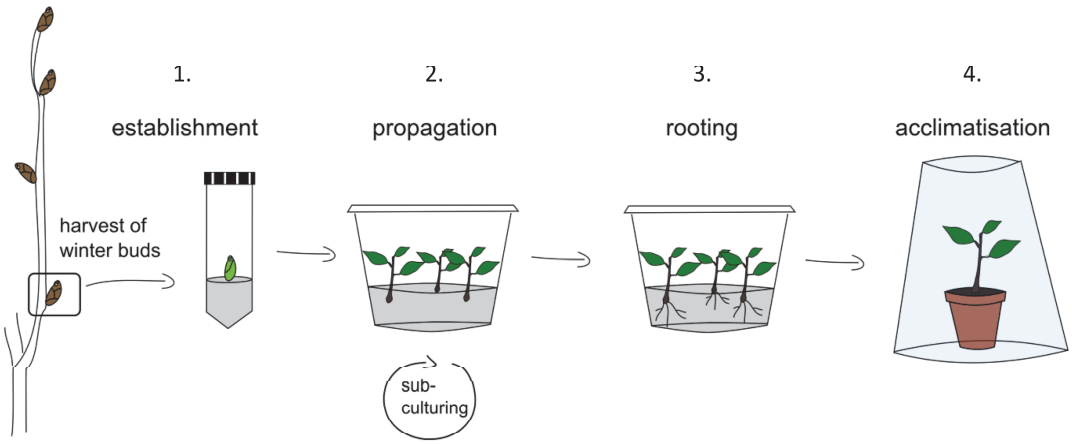


Fig. 2 Principle steps in propagation of *Prunus avium* microcuttings by in vitro culture.

While the propagation and acclimatization is successfully applied in commercial plant production, the rooting is still associated with high losses (for details see manuscript I, section 2.1). The rooting of mature cherry plants has been described as difficult compared to other tree species, very variable and dependent on genotype and age (Scaltsoyiannes et al. 2009). In accordance with these observations, large differences between genotypes and high fluctuations over the years have been observed during commercial production (for details see Quambusch et al. (2014), section 2.2).

In addition to the rooting difficulties, bacteria occurring in the plant material have been described as a limiting factor during in vitro propagation of wild cherry (Cornu and Michel 1987) and bacterial colonization has been observed in *Prunus avium* cultures of the silvaSELECT® collection used in this study. The possible linkage between the bacterial population and the propagation success *in vitro* will therefore be analyzed in this thesis.

1.2 Microbial Endophytes

1.2.1 Definition and general description

Plants are generally inhabited internally by a diverse microbial community including bacteria, archaea, fungi and phytoplasms (Hardoim et al. 2015). The plant-microorganism interactions include both mutualism and pathogenicity and depend on abiotic and biotic influences on the interaction partners (Hardoim et al. 2008). The term endophyte was first mentioned by the German botanist and mycologist Anton de Bary in 1866 and its definition has since changed several times in accordance with the increased understanding of endophytic lifestyle. While De Bary was still thinking of all microorganisms living inside the plant, Carroll (1986) restricted the use of the term to organisms that cause asymptomatic infections within plant tissues. As further studies revealed that pathogenicity and mutualism can lie very closely together and the same organism can switch between these lifestyles depending on the environmental conditions, a wider definition of the term was needed. Still up-to-date is the following definition that will be applied in this thesis:

> "[Endophytes are] *all organisms inhabiting plant organs that at some time in their life, can colonize internal plant tissues without causing apparent harm to their host"*
> (Petrini 1991)

This includes true symbionts as well as latent pathogens, microorganisms with both symptomless and saprophytic phases in their life cycle, bacteria and fungi that are colonizing the plant after a (dormant) stage in the soil or on the leaf/root surface as well as those persisting in the tissue over several plant generations.

There are several possibilities for the transfer of endophytes from one host to the next. Some endophytes persist in the seeds and are inherited by vertical transmission over several host plant generations. For example *Methylobacterium extorquens* has been detected in bud meristems, flowers and seed tissue of scots pine (Koskimäki et al. 2015),

and *Methylobacterium* and *Pantoea* species are frequently detected in seeds of diverse plant species (Mano and Morisaki 2008; Johnston-Monje and Raizada 2011). This enables a very close interaction or even a coevolution of the plant with its endophytic bacteria or fungi (Johnston-Monje and Raizada 2011). Several examples of coevolution are known for plant-fungal relationships, e. g. vertically and horizontally transmitted *Epichloë* producing herbivore deterring alkaloids in grasses (Schardl 1996; Morton et al. 2014) or the well-studied arbuscular mycorrhizal fungi colonizing over 92 % of all plant families studied (Wang and Qiu 2006). The most prominent example for symbiosis and coevolution in plant-bacteria relationships are the rhizobia who are able to fix atmospheric nitrogen only within special compartments of the root nodules of Fabaceae, enabling their host to grow with very low or even without nitrogen source in the soil (Oldroyd et al. 2011).

In contrast to vertical transmission the endophyte can infect new hosts horizontally by air, the soil, insect or other animal vectors. It is not rare to find endophytes rotating between different ecological niches, as seen for example in the life cycle of *Rhizobium leguminosarum,* where the bacterium migrates between the soil (as a heterotroph), the legume root-nodule (as an endosymbiont) and the cereal root (as a growth promoting endophyte) (Yanni et al. 2001). During the handling of plants by humans (e. g. by grafting, propagation by cuttings, in vitro propagation, the trimming of trees or ornamental plants) endophytes can be spread from one plant to the next and new microbes can be introduced by the skin surface, the soil or other contaminated surfaces and materials.

1.2.2 Bacterial endophytes

This thesis will concentrate on endophytes belonging to the domain of bacteria within the prokaryotic microorganisms. Compared to fungal endophytes the bacterial endophytes are far less studied. The interaction between bacteria and plants on molecular level has mostly been studied in the model organism *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens,* synonym *Agrobacterium radiobacter*) and in the legume-rhizobium symbiosis. The majority of bacterial endophytes that have been detected in the plant tissue are described as commensals with yet unknown functions in the plant. Although they are sometimes present in high numbers in the plant tissue, they do neither produce visible damage nor induce strong defense mechanisms in the plant (Reinhold-Hurek and Hurek 2011). Endophytic bacteria have been detected in virtually all plant tissues, including root, stem, leaves, meristematic tissues, flowers, seeds, and

berries e. g. by in situ hybridization (Pirttilä et al. 2000; Kukkurainen et al. 2004) and confocal laser scanning microscopy (Compant et al. 2008; Koskimäki et al. 2015). They can colonize both the intercellular and intracellular spaces and some move from the roots to the aerial plant parts by colonization of the xylem vessels (Compant et al. 2010). For some bacteria a positive influence has been described, others showed antagonistic effects on plants. A general classification to a plant-bacterium interaction group is still limited for most detected species as most of them have only been analyzed in one or few plant species, and are only rarely tested over a taxonomically wide spectrum of plant species (Hardoim et al. 2015).

1.2.3 Impact of bacterial endophytes on plants

Although our understanding is built on a rather small set of experimental conditions we can conclude that endophytic bacteria play crucial roles in the physiology of plants (Kozyrovska 2013). Some endophytes, called commensals, have no apparent effect on the plant but merely live on the metabolites produces by the plant. The second group confers a beneficial effect on the plant, either in form of a plant growth promotion or by protection against invading pathogens. A third group consists of latent pathogens persevering in the plant tissue until conditions are favorable for a systemic infection and disease development (Carroll 1988; Rosenblueth and Martínez-Romero 2006). This depicts that endophytes can have neutral, detrimental or beneficial effects depending on the abiotic environmental conditions, the bacterium and host genotype and developmental stage, and the interactions within the biome (Hardoim et al. 2015). A reduction of the host fitness can lead to a shift in the delicate balance in the endophytic community leading to disease expression by previously favorable bacteria or to saprophytic lifestyle during host senescence (Promputtha et al. 2007; Kozyrovska 2013). Bacteria can influence plant growth either directly, by providing the plant with compounds or by facilitating the uptake of nutrients from the environment, or indirectly, by the prevention of deleterious effects of pathogenic organisms (Glick and Penrose 1998).

Plant health

Bacteria can trigger defense mechanisms leading to induced systemic resistance (ISR) and confer a higher tolerance to pathogen infection. It has been described that, at the initial stage of interaction between beneficial microorganisms and plants, the immune response is still triggered, while the mutualists prevent later defense reactions of the plant (Compant et al. 2005). For the legume-rhizobium symbiosis the mechanism was analyzed on the molecular level showing an induction of immunity by microbe associated

molecular patterns (MAMP-triggered immunity) followed by a reprogramming of the defense mechanisms leading to a symbiotic interaction (Zamioudis and Pieterse 2012). In addition to the effect on pathogen resistance by ISR the bacterial endophytes can support plant health by the inhibition of pathogens. Endophytes produce a wide spectrum of antibiotics and volatile organic compounds (VOCs) suppressing the growth of their competitors, including plant pathogens (Ryan et al. 2008). Another indirect effect is the production of siderophores enabling the endophyte to protect the scarce iron-sources from their antagonists, thereby again suppressing the growth of potential plant pathogens. The reduction of abiotic stress through the degradation of the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) by the bacterial ACC deaminase or by reactive oxygen species (ROS) detoxification can additionally support plant health and studies on several crops indicate higher drought, chilling and salt tolerance after inoculation with endophytes (Hardoim et al. 2008; Hardoim et al. 2015). While the previously described mechanisms protect the plant against other microorganisms, some endophytes even produce insecticidal, nematicidal and antiviral compounds (Kaymak 2010).

Plant growth promotion

The morphology of the host can directly be influenced through the production of plant growth regulators by endophytes. Bacteria are able to produce a wide range of phytohormones that often play a crucial role in the bacteria-plant interaction. A classical example is the TI-plasmid of the pathogen *Rhizobium radiobacter* encoding key enzymes for the biosynthesis of the phytohormones auxin and cytokinin to stimulate cell proliferation and induce the formation of galls. While *Rhizobium radiobacter* manipulates the host-DNA to produce the hormones, several examples of endophytes are known produce phytohormones independently. Indole-3-acetic acid (IAA) biosynthesis is widespread among plant associated bacteria and different biosynthesis pathways have been identified (Spaepen et al. 2007). Bacteria use this phytohormone to interact with plants as part of their colonization strategy including phytostimulation, but also to circumvent basal plant defense mechanisms (Spaepen et al. 2007). Auxin producing bacterial strains have been reported for representatives of the genera *Pseudomonas*, *Enterobacter*, *Rhizobium*, *Bradyrhizobium*, *Bacillus*, *Methylobacterium*, *Rhodococcus*, *Acinetobacter*, and *Microbacterium* among many others (Tsavkelova et al. 2005). Ali et al. (2009) have confirmed a positive correlation between bacterial auxin production and an increased endogenous IAA level of plants (in this case *Triticum aestivum*); the amount produced is highly dependent on the host species, however (Long et al. 2008).

Other, less well studied influences on the phytohormone levels are the production of gibberellins, cytokinins and abscisic acid (Costacurta and Vanderleyden 1995; Salomon et al. 2013), metabolization of abscisic acid (Belimov et al. 2014), degradation of IAA (Spaepen et al. 2007), the induction of abscisic acid and salicylic acid production in the host plant (Wang et al. 2015), and the modulation of ethylene levels in the plant tissue (Hardoim et al. 2008). By these means endophytes are able to induce biomass production, shoot and root growth and even delay flower senescence in cut flowers (Ali et al. 2012).

Endophytic bacteria can also lead to plant growth promotion by an increase of nutrient availability. The ability to solubilize phosphate and other minerals is widely spread among the bacterial domain (Hilda and Fraga 1999). Diazotrophic bacteria, e. g. the plant growth promoting bacterium (PGPB) *Azospirillum brasilense* colonizing maize roots, are able to fix atmospheric nitrogen and supply the plant with nitrate (Faleiro et al. 2013).

1.2.4 Role of endophytes in in vitro culture

Although this has been the common understanding for several decades, today it has to be assumed that plant in vitro cultures are never entirely free of microorganisms (Herman 1990). During the establishing of in vitro cultures the plant tissue is surface sterilized. Endophytic bacteria and fungi living inside the tissue will survive this process and persist in the material (Marino et al. 1996). Before the 1990s bacteria observed in the cultures were by most scientists considered to be ‘contaminants’, introduced to the cultures by human handling of the samples, and the final aim was elimination of the microorganisms to obtain a ‘sterile’ culture (Leifert et al. 1991). The focus was on the negative influences that especially bacteria can have on the vitality of tissue culture, for example the browning and early senescence of plant material (Liu et al. 2005) or the vigorous growth of bacteria on the surface of culture media eventually leading to overgrowth of the plant (Leifert and Cassells 2001).

The presence and function of endophytes in tissue cultures has been increasingly under investigation during the last years (also see introduction of Publication 1 and 2). Several studies have demonstrated the presence or even positive effect of endophytic bacteria in tissue cultures (Thomas et al. 2008; Dias et al. 2008; Abreu-Tarazi et al. 2010; Jimtha et al. 2014), including tissue cultures of trees (Izumi et al. 2008; Zaspel et al. 2008; Scherling et al. 2009; Donnarumma et al. 2011; Quambusch et al. 2014). The endophyte species composition and plant genotype together with tissue culture conditions seem to have a strong impact on the development of a negative or positive interaction (Pirttilä et al.

2008; Quambusch et al. 2014). The presence of bacterial endophytes in the cultures is often visually observable after changes in culture conditions, e. g. after the transfer to rooting medium. In most cases the bacteria occur as clouds in the culture medium at the base of the microshoot (Fig. 3 AB), less often on the surface of the culture medium in contact with the stem or leaves of the explants (Fig. 3 C). The practical experience in our in vitro culture laboratory with *Prunus avium*, several cultivars of *Malus* and a diverse group of other plants is that the visual observation of endophytes is not necessarily connected with growth inhibition or other negative effects on the plant. In some cases a browning of the tissue in connection with plant senescence was observed during apparent growth of bacteria, as described by Pirttilä et al. (2008). It is not known whether the endophytes are involved in browning by inducing the senescence, or the endophytes simply take over the already senescing tissue (Pirttilä et al. 2008).

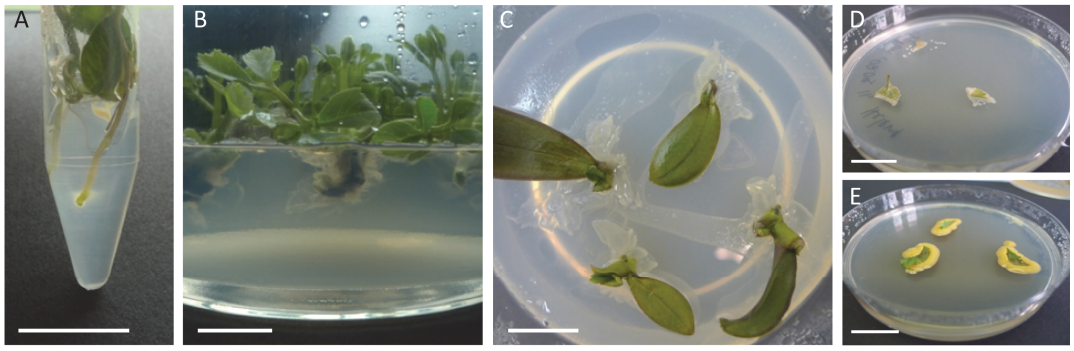


Fig. 3 Visual observation of endophytic bacterial growth in different plant tissue culture materials. (A) Tissue culture of *Solanum* sp. with bacteria growing out of the basal part of the microshoot (B) Endophytes emerging from the submerged parts of *Helleborus* microshoots (C) bacteria of *Phalaenopsis* tissue culture growing on the surface of the medium (D) Leaves of *Prunus avium* genotype Neptun on bacterial culture medium with white colonies of isolate N-I-2 (*Rhodopseudomonas palustris*) (E) Leaves of *Prunus avium* genotype Demeter on bacterial culture medium with yellow colonies of isolate D-I-1 (*Microbacterium testaceum*).

From the previously discussed literature it can be concluded, that endophytes can have positive effects during in vitro culture of plants. At the same time we have to keep in mind that "contaminations" do cause serious troubles in commercial in vitro culture propagation (Kulkarni et al. 2007). The shape of the interaction with specific endophytes is not fixed and can change if biotic or abiotic factors are altered. Keeping the balance of the total microbial community seems to be more promising for successful tissue culture propagation than the suppression or elimination of bacteria:

> *"It seems there is an equilibrium of endophytes and plants that under certain circumstances may be unbalanced to the detriment of one of the partners"*
> (Rosenblueth and Martínez-Romero 2006)

1.2.5 Detection of bacterial endophytes

It is common understanding that only a small percentage of bacteria are cultivable by standard techniques. The portion of "as yet cultivated" bacteria has been estimated to be below 1 % (Vartoukian et al. 2010) or ranging from 1-10 % (Schleifer 2004). A true understanding of the physiology and function of a bacterial species requires the study of living cells. With the aim to get the closest approach to the total bacterial community it is therefore necessary to cover both the cultivable and non-cultivable fraction of endophytes.

The culture-dependent detection starts with the plating of plant material on bacterial culture media to obtain bacterial colonies. In the following process a pure culture of the bacteria is obtained, the DNA is extracted and a target-gene, most commonly the 16S rDNA, is amplified by PCR and sequenced for the taxonomic assignment (see Fig. 4 and manuscript II, section 2.2). The most critical step of this method is the selection of suitable bacterial culture conditions. To enable the growth of a high diversity of bacteria the use of media with low nutrient content, the extension of the incubation time and the simulation of natural environmental conditions was proposed by Vartoukian et al. (2010). Several groups tested innovative new techniques for the culture of "as yet uncultivables", e. g. the use of a hollow fibre membrane chamber (Aoi et al. 2009) or magnetic nanoparticle mediated isolation proposed by Zhang et al. (2015). An example for the successful cultivation of previously uncultivable *Mycobacterium* spp., present in all analyzed *Prunus avium* genotypes, is given in manuscript III.

For the culture-independent detection of bacteria the DNA is extracted directly from the plant material. Subsequent steps are the amplification of the target gene by PCR and the establishment of a clone library containing the amplification products (Fig. 4). The plasmids of the clone library can either directly be sequenced or an intermediate step can be included to reduce the number of necessary sequencing runs. One possibility is the conduction of an amplified ribosomal DNA restriction analysis (ARDRA) followed by the grouping of the clones based on their restriction pattern, as applied in this thesis (see Fig. 4 and introduction of manuscript II). Using this method only one clone for each observed restriction pattern has to be sequenced and compared to database entries for the taxonomic placement. The technical possibilities to analyze the unculturable fraction of the endophytic bacteria have been subject of huge changes during the last years by the invention and dissemination of next generation sequencing which are discussed in detail in 3.1 and 3.2.

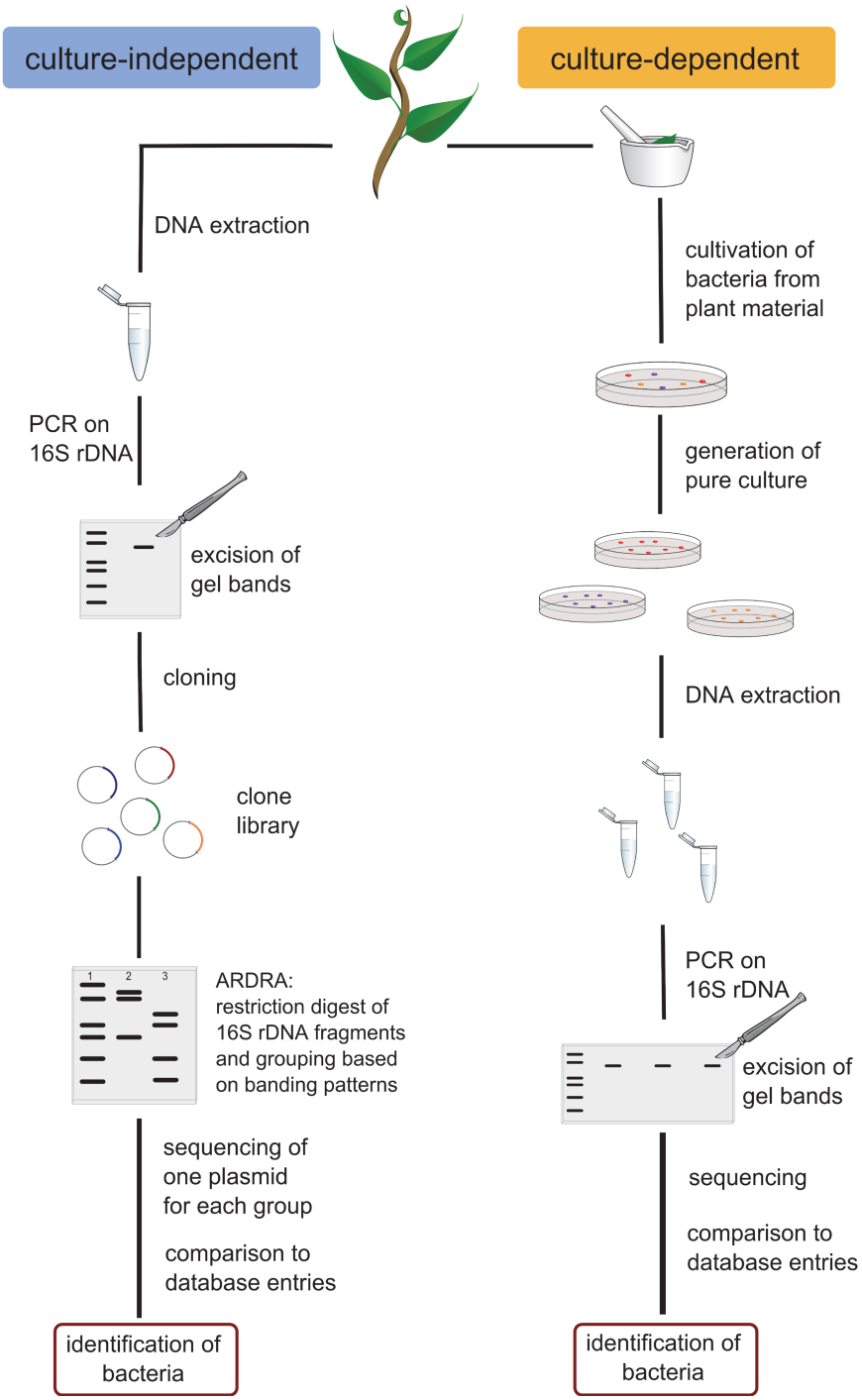


Fig. 4 Workflow for the detection of endophytes by culture-dependent and culture-independent method as used in this thesis.

The most frequently used sequence for phylogenetic studies is the approximately 1500 bp long and highly conserved 16S rDNA encoding the small subunit of the ribosome of bacteria (Clarridge 2004; Woo et al. 2008). Because this sequence is used in most studies the databases (e. g. NCBI GenBank or ribosomal database project (RDP)) cover a wide spectrum of bacterial sequences and identification of rare or non-cultivable species is

made possible. Identification by 16S rDNA sequences is limited when it comes to the differentiation of closely related species and the classification to species or subspecies level. In this case sequencing of the 16S-23S internal transcribed spacer (ITS) can be additionally used to ensure and refine the phylogenetic placement (Nesme and Normand 2004). Although widely used in the identification of fungus species, ITS sequences of bacteria are far less covered in the databases and for rare or non-cultivable species often no entry of the same species can be found.

1.3 Cooperation project: "New possibilities of characterization, regulation and utilization of bacterial endophytes in in vitro propagation systems of wild cherry (*Prunus avium* L.)"

The studies in this thesis were performed as part of a cooperation project with the title "New possibilities of characterization, regulation and utilization of bacterial endophytes in in vitro propagation systems of wild cherry (*Prunus avium* L.)" between the Leibniz Universität Hannover, Woody Plant and Propagation Physiology Section and the Institut für Pflanzenkultur (IFP) based in Solkau, Schnega. The project was financed by the German Federal Ministry for Economic Affairs and Energy (BMWi) as part of the funding program ZIM (Zentrales Innovationsprogramm Mittelstand) that supports small and medium-sized enterprises which want to develop or improve innovative products, processes or technical services [grant number KF2508004AJ0].

The IFP is working in the in vitro production, acclimatization to greenhouse conditions and field cultivation of plants. They combine research and application to tissue culture methods and optimize culture protocols or produce young plants for their customers. Another key activity of the IFP is the development of microbial products in form of soil-adjuvants to improve plant growth on suboptimal substrates or sites. The propagation of *Prunus avium* genotypes for the silvaSelect© Gehölze GmbH was of major commercial relevance during the last years. In the year 2010, before the start of the cooperation project, 69 % losses of plants were observed during the rooting and acclimatization of *Prunus* microcuttings. At the same time the increased appearance of bacteria was observed during the micropropagation process and financial losses due to endophytes were taken into account.

The aim of the project was to identify bacterial endophytes in *Prunus avium* tissue culture and analyze their function in a holistic approach looking both on possible negative and positive effects of the bacteria. In contrast to the often used elimination of

bacteria in tissue cultures the focus was put on the evaluation of the total bacterial community to obtain information on their balance or imbalance during tissue culture propagation. The main hypothesis was that the observed variability in the propagation success between genotypes was related to a different bacterial community, and that this community can be influenced in a manner favorable for plant growth. The three main objectives of the cooperation project were:

- Development of an economically efficient propagation protocol for the silvaSelect© genotypes
- Production of high quality plant material
- Isolation, identification and characterization of bacteria from tissue culture

The development of a new marketable product on the basis of bacteria with positive effects on plant growth was a further goal but was left optional because the feasibility was unpredictable at project start.

1.4 Thesis objectives

While several studies unraveled numerous functions of endophytes in the plant there is still little known about their behavior during in vitro culture. Previously published works in the majority focused on an inventory of the bacterial population or on single PGPB strains. The aim of this thesis was to study the dynamics of the endophytic population of *Prunus avium* in vitro cultures to obtain a better understanding of the positive, neutral and negative influences that endophytic bacteria can have during in vitro propagation of plants. Additionally, the rooting protocol, limiting commercial production of *Prunus avium,* should be optimized and the obtained knowledge brought together to obtain an improved protocol for the micropropagation of *Prunus avium*.

The detailed objectives of this thesis were as follows:

1. Development of an improved protocol for the rooting of microshoots to enable the propagation of a wide spectrum of *Prunus avium* genotypes in commercial scale.
2. The identification of bacterial endophytes in *Prunus avium* genotypes with differing propagation success on the genus level.
    a. Establishment of laboratory protocols for the identification of bacterial isolates (culture-dependent method) and for the identification of the total bacterial community *in planta* (culture-independent method)

   b. Taxonomic assignment of bacterial isolated obtained in our group and by the project partners.

3. Characterization of the detected bacteria regarding their impact on *Prunus avium* during micropropagation.

   a. Evaluation of the growth promoting potential of the detected and isolated bacterial strains by comparison to database entries and references of other studies.

   b. Screening of bacterial isolates for their ability to produce auxin and siderophores, metabolize organic phosphates and fix atmospheric nitrogen. (Comment: These aspects were part of the master thesis of Kristin Haller.)

4. Quantification of the most abundant bacterial strains to gain knowledge about the fluctuations of endophytes during in vitro culture.

   a. Establishment of quantitative PCR (qPCR) protocols for the most abundant bacterial strains.

   b. Comparison of the bacterial amount present in different genotypes, plant organs, during different in vitro culture phases and at different sampling times.

   c. Evaluation of the influence of de novo organogenesis on the bacterial community by regeneration of adventitious shoots and quantification of the bacterial strains.

5. Utilization of bacterial endophytes to improve the rooting of *Prunus avium.*

   a. Inoculation of difficult-to-propagate *Prunus avium* genotypes with bacterial isolates from easy-to-propagate genotypes during in vitro rooting.

2 Publications and Manuscripts

2.1 Improved in vitro rooting of *Prunus avium* microshoots by a dark treatment and an auxin pulse.

Mona Quambusch[a], Silke Gruß[b], Toni Pscherer[a], Traud Winkelmann[a], Melanie Bartsch[a]

[a]Leibniz Universität Hannover, Institute of Horticultural Production Systems, Woody Plant and Propagation Physiology Section, Herrenhäuser Str. 2, 30419 Hannover, Germany, bartsch@baum.uni-hannover.de, tel: 0049 511 762 4539, fax: 0049 511 762 3608

[b]Institut für Pflanzenkultur e. K., Solkau 2, 29465 Schnega

Type of authorship:	First author
Type of article:	Research article, short communication
Contribution to the article:	Planned and performed 50 % of experiments Analyzed all data Prepared all figures Wrote the paper
Contribution of other authors:	Toni Pscherer performed 50 % of rooting and acclimatization experiments in his bachelor thesis[2] Silke Gruß supplied data from commercial productin Traud Winkelmann and Melanie Bartsch contributed to experimental design, data analysis and writing the paper
Journal:	Scientia Horticulturae
Impact factor:	1.365
Date of manuscript submission:	15.03.2016, currently under review
DOI:	-

[2] Pscherer, Toni (2013) Optimierung der Bewurzelung und Akklimatisierung von *Prunus avium* In-vitro-Sprossen. Bachelor's thesis, Leibniz Universität Hannover

Title

Improved in vitro rooting of Prunus avium microshoots by a dark treatment and an auxin pulse.

Authors

Mona Quambusch[1], Silke Gruß[2], Toni Pscherer[1], Traud Winkelmann[1], Melanie Bartsch[1]

Abstract

The influence of a four days dark treatment and a 48 h IBA pulse on in vitro root formation of *Prunus avium* microshoots was examined. Both treatments led to significantly higher rooting percentages in the majority of the six analyzed genotypes. While the IBA pulse led to disproportional growth of roots compared to shoots, the plantlets of the dark treatment during root induction grew healthy and were visually not distinct from the control plants. Further data collected during commercial production of 28 genotypes verified the improved root induction by a dark treatment. In addition, the importance of in vitro rooting for the successful transfer to greenhouse conditions was confirmed. While plantlets with 1 to 3 roots already showed improved growth in the greenhouse the well rooted plantlets (>3 roots) had a significantly higher survival and improved growth during acclimatization than unrooted microshoots.

Affiliations

[1]Leibniz Universität Hannover, Institute of Horticultural Production Systems, Woody Plant and Propagation Physiology Section, Herrenhäuser Str. 2, 30419 Hannover, Germany, bartsch@baum.uni-hannover.de, tel: 0049 511 762 4539, fax: 0049 511 762 3608

[2]Institut für Pflanzenkultur e. K., Solkau 2, 29465 Schnega

Keywords

Acclimatization; adventitious rooting; cherry; commercial in vitro production; IBA; micropropagation

Abbreviations

IAA: indole-3-acetic acid; IBA: indole-3-butyric acid

1. Introduction

The timber of wild cherry (*Prunus avium* L.) is highly valued in Europe due to its reddish color, comparable to tropical trees like mahogany, and the firmness of the wood, combined with fast and fine grained growth (Kobliha 2002; Russell 2003). It is used for the production of solid wood furniture and as veneer in interior fittings (Schmid 2006). Trees with optimized growth characteristics, such as fast growth, straight stem and fine grains have been selected and are distributed under the trademark silvaSELECT® (Janßen et al. 2010). Vegetative propagation of these certified genotypes via in vitro propagation enables high multiplication rates and stable clonal plant material (Meier-Dinkel et al. 2007). To ensure genetic diversity in forestry, a set of 31 genotypes was registered and is propagated in vitro for tree nurseries (Janßen et al. 2010). In commercial production, however, severe losses are observed during in vitro rooting and acclimatization. The propagation success is strongly dependent on the genotype and shows a high fluctuation over time (C. Schneider, personal communication). From 2009 until 2012 the genotypes used in this study showed 20 % - 61 % losses during rooting in commercial production making an optimization of this cultivation step necessary for cost-effective plant production.

The formation of adventitious roots is affected by light energy and in many plants induced by darkness (George and Davies 2008). A dark treatment has previously been used to improve adventitious root formation ex vitro, e. g. Klopotek et al. (2010) could strongly improve rooting of petunia cuttings by dark exposure and reported an accelerated formation of root meristems in a histological analysis. For in vitro propagation Monteuuis and Bon (2000) and Druart et al. (1982) reported a significant increase in the proportion of rooted microshoots of *Acacia mangium* and *Malus domestica*, respectively, in darkness compared to standard lighting conditions.

De Klerk et al. (1992; 1995) showed in their studies that auxin is required to induce roots but inhibits the outgrowth of root primordia and formation of roots at later stages of development. On the basis of these results adventitious root formation was divided into three stages: a dedifferentiation phase in which cells are not sensitive for auxin application (0h-24h after cutting), an induction phase where auxin initiates first cell divisions (24h-96h) and a differentiation phase (96h +) with the formation of root primordia that is inhibited by auxin supplementation (De Klerk et al. 1999). In scientifically and commercially used protocols for rooting of *Prunus avium* microshoots IBA is added for the total duration of the rooting phase (Meier-Dinkel 1986). This

procedure is sufficient for many genotypes but leads to low numbers of rooted plants for others (Quambusch et al. 2014).

To ensure genetic diversity of forest sites it is important to successfully propagate the maximum number of genotypes available instead of concentrating on those that are easy-to-propagate. The overall objective of this study was to optimize the in vitro rooting phase to enable a successive acclimatization of a high number of *Prunus avium* genotypes which can be applied for commercial production. In detail the aims were to test (1) the influence of a dark treatment during the first four days of the rooting phase and (2) the effect of an auxin (IBA) pulse during the first 48 h on the formation of adventitious roots of *Prunus avium* microcuttings. To our knowledge these two parameters have not been tested for *Prunus avium* tissue culture before.

2. Materials and Methods

2.1 Plant material

For the present study, six *Prunus avium* genotypes of the silvaSELECT® selection with varying propagation success (here defined as rooting rate × acclimatization rate, given as a percentage) were selected based on data from commercial production for the years 2009–2012 (data collected at the Institut für Pflanzenkultur, Germany). Genotypes Fama and Achilleus were difficult-to-propagate genotypes, Asteria and Apollo showed high fluctuation over the years, resulting in medium propagation success and Neptun and Demeter were easy-to-propagate genotypes (for details on propagation success data see (Quambusch et al. 2014)). The genotypes were formerly selected at the Northwest German Forest Research Institute (NW-FVA) for their growth parameters, and in vitro cultures had been established from surface-sterilized winter buds and propagated for 4-16 years via axillary shoots.

2.2 In vitro culture conditions

Shoots were propagated on MS medium (Murashige and Skoog 1962) containing 3 % (w/v) sucrose and 0.6 % Plant Agar (Duchefa, Haarlem, The Netherlands), supplemented with 2.22 µM benzylaminopurine, 0.49 µM indole-3-butyric acid and 0.29 µM gibberellic acid-3 and adjusted to pH 5.8. Each 500-ml plastic vessel contained 80 ml of culture medium and 10 plants. The cultures were incubated at 24 °C under a 16-h photoperiod (40–56 µmol m^{-2} s^{-1}) and subcultured every 5 weeks. To induce rooting the shoots were transferred to rooting medium containing 4.9 µM indole-3-butyric acid as

the sole plant growth regulator (PGR) and macronutrients reduced to 1/3 MS, all other aspects were the same as described for the propagation medium.

2.3 Rooting experiment

To optimize the rooting of the six *Prunus avium* genotypes three treatments were compared: Treatment 1 was the control treatment under the conditions described above. In treatment 2 the shoots were placed on rooting medium and cultured in darkness for four days prior to transfer to standard culture conditions. For treatment 3 the shoots were transferred to rooting medium with a 10x higher concentration of IBA (49 µM) for 48 h prior to the transfer on rooting medium without any PGR supplementation. 100 shoots were treated per variant in ten vessels (replicates) each. The experiment was repeated three times (November 2012, January 2013 and October 2013). The percentage of shoots with adventitious roots was evaluated after four weeks on rooting medium.

Plants in commercial production were propagated with the standard protocol as stated above (2.2), except that 15 shoots were cultured in one vessel during propagation and 20 during rooting. To induce adventitious rooting the standard protocol was used from 2009 to 2012. From 2013 to 2015 all shoots were incubated in darkness for the first 7 days on rooting medium, but with Plant Agar being replaced by Gelcarin (0.7 %, Duchefa, Haarlem, The Netherlands). The number of analyzed genotypes ranged from 7 to 28 and the number of analyzed plants ranged from 1 900 to 286 720 between propagation times.

2.4 Acclimatization

After 4 weeks on standard rooting medium, in vitro shoots of the *Prunus avium* genotypes Neptun, Apollo and Achilleus were sorted into three groups prior to acclimatization: group 1 without roots; group 2 with 1-3 roots; group 3 with > 3 roots. The plantlets were transferred to potting mix containing white peat and ¼ perlite with a grain size 1-7.5 mm (“Steckmedium”, recipe #300, Klasmann-Deilmann, Geeste, Germany) in 4 cm x 4 cm pots and kept under a foil tunnel with floor heating at 22°C and nearly 100 % humidity in a greenhouse with a 16 h photoperiod provided by additional lighting during the winter months. After 2 weeks the humidity was slowly reduced and after 3-4 weeks the plants were transferred to a greenhouse with a minimum temperature of 18 °C (heating setpoint). From then on, plantlets were fertilized once a week with 0.07 % Ferty® MEGA 3 (Planta Düngemittel, Regenstauf, Germany). The acclimatization rate was evaluated after 7 weeks. The experiment was conducted four times; and the total number of plants used is depicted in Fig. 1.

2.5 Statistical analysis

All statistics were performed with the program R-3.2.2 (R Development Core Team 2008). An ANOVA was conducted to describe variation among treatments and genotypes as well as the interaction between them. Differences between control and treatment were tested for significance using Dunnett's test based on generalized linear models for each genotype using logit transformation (McCullagh and Nelder 1989). In the rooting experiment the culture vessel was the randomized unit and the test was performed with percentages of rooted shoots in each culture vessel except for experiment repetition two where all vessels were treated as one unit (information on culture vessels were not available).

3. Results and Discussion

3.1 Importance of in vitro rooting on acclimatization success

In a preliminary experiment we evaluated the effect of in vitro rooting on the survival of plants during acclimatization to greenhouse conditions. Differences in plant growth were clearly visible four weeks after transfer to the greenhouse (Fig. 1 A). The effect was measurable after the plants were fully acclimatized to the greenhouse, seven weeks after the transfer. A significant effect of the factor rooting (unrooted plants, plants with 1-3 roots and plants with > 3 roots) was detected by ANOVA ($p=2.28x10^{-05}$). Analysis of the acclimatization success for each genotype verified a significant difference between unrooted microcuttings and microcuttings with > 3 roots for Neptun and Achilleus (Fig. 1 B). The group of microcuttings with 1-3 roots positioned in between unrooted and well-rooted plants with an average of 48 % (±35 %), 32 % (±26 %) and 31 % (±17 %) surviving plants for genotypes Neptun, Apollo and Achilleus, respectively (Fig. 1 B).

Although some studies indicated an influence of different growth conditions during rooting on acclimatization (e.g. Muna et al. 1999), to our knowledge the direct effect of the shoot rooting status on acclimatization was not tested before for Prunus avium. De Klerk et al. (1999) stated that it is preferable to root small microcuttings in vitro and transfer them to soil after roots have been formed as the plantlets increase in size and become more robust during this cultivation step. In agreement with our results this emphasizes the importance of an efficient protocol for the rooting of microshoots to ensure a successful transfer to the greenhouse and to minimize losses during the acclimatization phase. Our data additionally indicate that for Prunus avium it is advisable to use only plantlets with more than three roots for acclimatization.

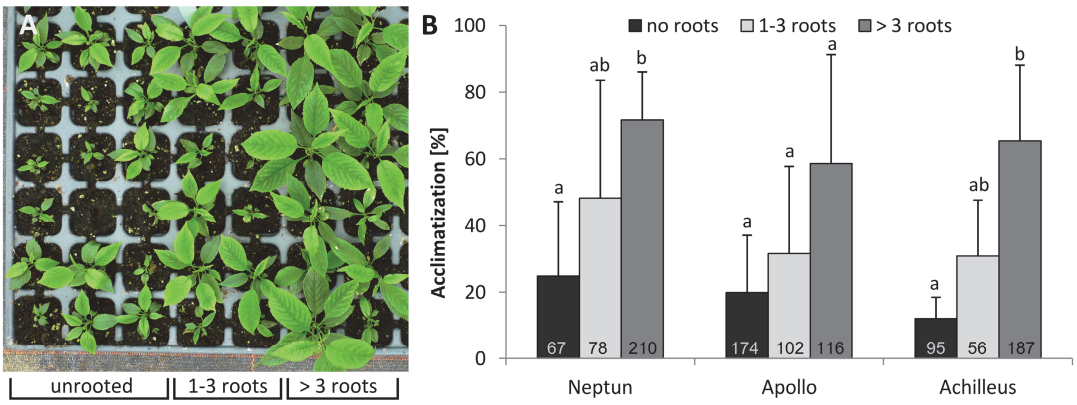


Fig. 1 Effect of in vitro rooting on the acclimatization success. (A) Plantlets four weeks after transfer to the greenhouse, genotype Neptun. (B) Acclimatization rate seven weeks after transfer to the greenhouse. Different letters indicate significant differences between the groups within one genotype by Tukey's range test ($P < 0.05$); Bars = mean of four repetitions; Error bar = SD; n = 4 repetitions, number of total individuals are given in the bars.

3.2 Induction of roots by an IBA pulse

To improve the rooting of *Prunus avium* microshoots the effect of a 4 days dark treatment and an IBA pulse for 48 h was tested. Applying an IBA pulse led to a significant increase of rooting percentage in five of six genotypes tested and the strongest increase was measured for Achilleus with 86 % (± 9 %) rooted plants compared to 65 % (± 22 %) in the control (Fig. 2). However, this treatment did not lead to strong and healthy plants needed for the acclimatization to greenhouse conditions.

In Fig. 3 examples of rooted microshoots of all treatments are depicted and the IBA pulse treated plants (Fig. 3 g-i) showed disproportionally big roots with small shoots. Thus, an optimization of the concentration or duration of the application of IBA would be needed to use the beneficial effect of the pulse treatment on rooting.

Label et al. (1989) hypothesized that exogenous IBA is only needed to trigger the developmental condition for rooting, whereas other studies suggest a positive effect of auxin during the early induction phase, but a repressive effect on root development thereafter (De Klerk et al. 1999). Although our results are in line with these findings, the use of a pulse treatment instead of continuous IBA application during rooting of *Prunus avium* can only be recommended with limitations: First, the treatment involves one additional transfer of the microshoots to fresh medium leading to much higher costs for material and labour during commercial production, and second the problem of overdosage of IBA as seen in Fig. 3 g-i needs to be solved. The extra-subculturing might

be saved, if - instead of IBA - IAA was used which rapidly is degraded under light or riboflavin is added that facilitates the inactivation of IBA in the light (Krieken et al. 1992; De Klerk et al. 1999).

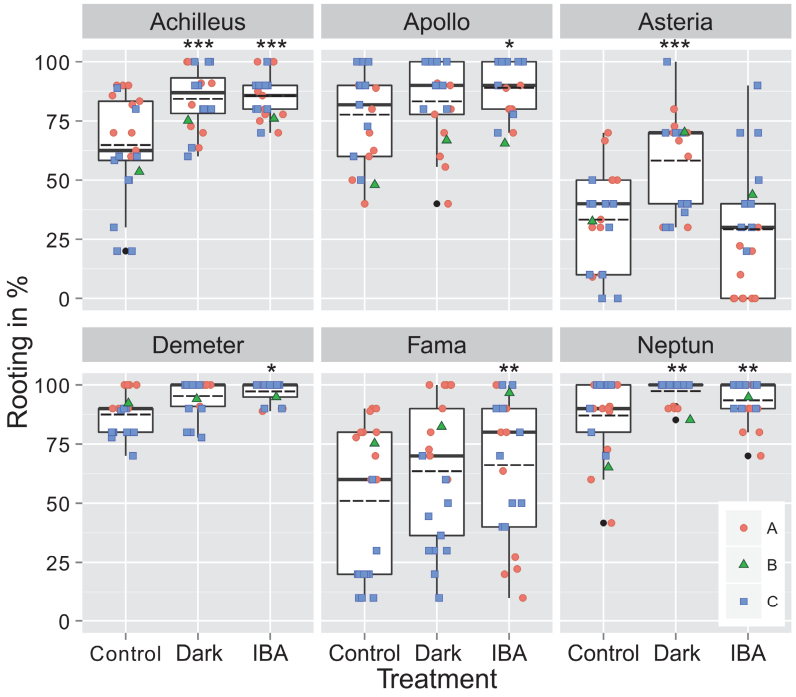


Fig. 2 Comparison of the percentage of rooted plants produced using the standard protocol (control), a four days dark treatment (dark) or an 48 h IBA pulse (IBA). The 3 repetitions are depicted as red dots (A), green triangle (B) and blue squares (C), each dot represents one vessel (except for B, representing the whole experiment). Boxplot with additional information on the mean (dashed line). Asterisks indicate significant differences between the treatments and the corresponding control by Dunnett's test (*, ** and *** indicate $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively); n = 20-21.

3.3 Induction of roots by dark treatment

The dark treatments proved successful in inducing a higher number of rooted plants (Fig. 2). The increase through dark treatment was strongest for genotypes Achilleus with a mean of 65 % (± 22 %) in the control and 84 % (± 13 %) with dark treatment and Asteria with an increase from 33 % (± 20 %) to 58 % (± 21 %) with dark treatment. Data collected during commercial production, where the protocol was changed to a seven days dark treatment in 2013, underline the results. In the years 2009-2012 the average rooting rate was 59 % (± 9 %) and from 2013 to 2015 the rate was clearly higher with 79 % (± 7 %) rooted plants (mean ± SD over the propagation sets and the genotypes, for details see 18.3). Although other changes in the protocol (e.g. a change in the solidifying agent of

the rooting medium) might have influenced the data, this is clear illustration of the applicability and feasibility of the dark treatment in commercial practice.

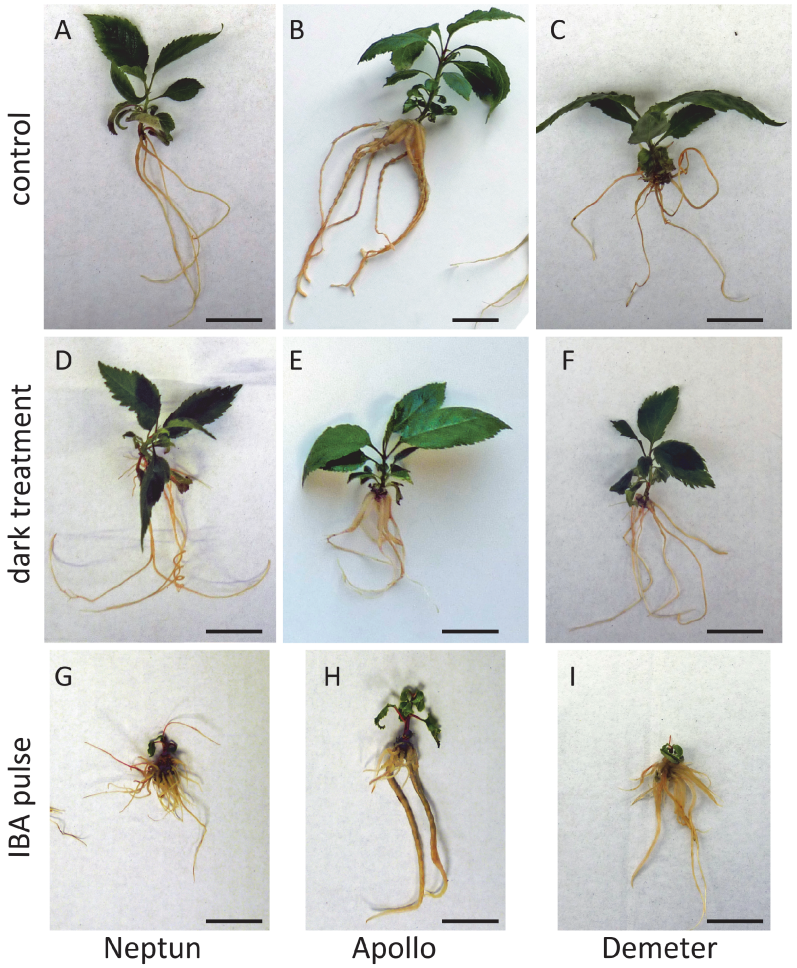


Fig. 2 Examples of rooted microshoots of *Prunus avium* genotypes Neptun, Apollo and Demeter after four weeks on rooting medium. (A) – (C) cultivated with standard protocol; (D) – (F) cultivated on standard medium with 4 days dark treatment; (G) – (I) cultivated with a 48 h IBA pulse before transfer on hormone free rooting medium. Bars represent 2 cm.

A beneficial effect of darkness on rooting, as seen in our results, is in accordance with findings of Krieken et al. (1992) who described a higher rooting percentage after five days darkness during the induction phase in apple. In a study of Monteuuis and Bon (2000) even the culture in total darkness through the whole rooting procedure proved positive for rooting of *Acacia mangium*. The same was reported for *Prunus serotina* where total darkness (Fuernkranz et al. 1990) and a four days dark treatment (Espinosa et al. 2006) resulted in highest rooting percentages. In our materials and experimental conditions the extension of the dark treatment to seven days did not improve the rooting percentage further (data not shown) and in a trial during commercial propagation of *Prunus avium* a

nine days dark treatment had a negative effect on rooting (data not shown). The high variability of the data between repetitions, especially for genotype Fama, could be related to fluctuations in the endophytic community as shown in a previous publication (Quambusch et al. 2014).

3.4 Conclusions

Both IBA pulse and four days dark treatment led to an increase in the percentage of rooted plants of *Prunus avium* microshoots. While the IBA pulse lead to disproportional growth of roots compared to shoots, the plantlets kept dark during root induction grew healthy and were visually not distinct from the control plants. As the dark conditions can easily be applied and the effect was highly reproducible in this study, the dark treatment can be recommended for commercial in vitro propagation of a broad set of *Prunus avium* genotypes. Instead of genotype-dependent optimization of propagation protocols, the overall application of the described dark treatment led to a higher production of rooted plants. Thus, the transfer of technological knowledge to practice, which is often limited by unreproducible effects or technical limitations under commercial conditions, was successful in this study.

Acknowledgements

The authors thank technicians Bärbel Ernst, Ewa Schneider and Friederike Schröder for their support.

Financial support of the German Federal Ministry of Economics and Technology within the program PRO INNO Grant no. KF2508004AJ0 is gratefully acknowledged.

M.Q. is a member of the graduate school WeGa PhD funded by the German Federal Ministry of Education and Research and the Ministry for Science and Culture of Lower Saxony.

References

De Klerk GJ, Keppel M, Brugge J Ter, Meekes H (1995) Timing of the phases in adventitous root formation in apple microcuttings. J Exp Bot 46:965–972. doi: 10.1093/jxb/46.8.965

De Klerk GJ, Ter Brugge J (1992) Factors affecting adventious root formation in microcuttings of *Malus*. Agronomie 12:747–755.

De Klerk GJ, van der Krieken W, de Jong J (1999) Review: The formation of adventitious roots: New concepts, new possibilities. Vitr Cell Dev Biol - Plant 35:189–199. doi: 10.1007/s11627-999-0076-z

Druart P, Kevers CL, Boxus P, Gaspar T (1982) In vitro promotion of root formation by apple shoots through darkness effect on endogenous phenols and peroxidases. Zeitschrift für Pflanzenphysiologie 108:429–436. doi: 10.1017/CBO9781107415324.004

Espinosa AC, Pijut PM, Michler CH (2006) Adventitious shoot regeneration and rooting of *Prunus serotina* in vitro cultures. HortScience 41:193–201.

Fuernkranz HA, Nowak CA, Maynard CA (1990) Light effects on in vitro adventitious root formation in axilary shoots of mature *Prunus serotina*. Physiol Plant 80:337–341.

George EF, Davies W (2008) Effects of the Physical Environment. In: George EF, Hall MA, De Klerk G-J (eds) Plant Propagation by Tissue Culture, 3rd Editio. Springer, pp 424–463

Janßen A, Meier-Dinkel A, Steiner W, Degen B (2010) Forstgenetische Ressourcen der Vogel-Kirsche/ Forest genetic resources of wild cherry. Forst und Holz 65:19–24.

Klopotek Y, Haensch K-T, Hause B, Hajirezaei M-R, Druege U (2010) Dark exposure of petunia cuttings strongly improves adventitious root formation and enhances carbohydrate availability during rooting in the light. J Plant Physiol 167:547–54. doi: 10.1016/j.jplph.2009.11.008

Kobliha J (2002) Wild cherry (Prunus avium L.) breeding program aimed at the use of this tree in the Czech forestry. J For Sci 48:202–218.

Krieken WM Van Der, Breteler H, Visser MHM, Jordi W (1992) Effect of light and riboflavin on indolebutyric acid-induced root formation on apple in vitro. Physiol Plant 85:589–594.

Label P, Sotta B, Miginiac E (1989) Endogenous levels of abscisic acid and indole-3-acetic acid during in vitro rooting of Wild Cherry explants produced by micropropagation. Plant Growth Regul 8:325–333. doi: 10.1007/BF00024663

McCullagh P, Nelder JA (1989) Generalized linear models, Vol. 37. CRC press

Meier-Dinkel A (1986) In vitro Vermehrung ausgewählter Genotypen der Vogelkirsche (*Prunus avium* L.). Allg Forst- u J-Ztg 157:139–144.

Meier-Dinkel A, Steiner W, Artes O, Hosius B, Leinemann L (2007) Die silvaSELECT-Vogelkirschen- Klonmischung „Escherode I“. AFZ-Der Wald 5:246–247.

Monteuuis O, Bon MC (2000) Influence of auxins and darkness on in vitro rooting of micropropagated shoots from mature and juvenile *Acacia mangium*. Plant Cell Tissue Organ Cult 63:173–177. doi: 10.1023/A:1010611126950

Muna A-S, Ahmad A-K, Mahmoud K, Abdul-Rahman K (1999) In vitro propagation of a semi-dwarfing cherry rootstock. Plant Cell Tissue Organ Cult 59:203–208. doi: 10.1023/A:1006444925445

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497.

Quambusch M, Pirttilä AM, Tejesvi M V., Winkelmann T, Bartsch M (2014) Endophytic bacteria in plant tissue culture: differences between easy- and difficult-to-propagate *Prunus avium* genotypes. Tree Physiol 34:524–33. doi: 10.1093/treephys/tpu027

R Development Core Team (2008) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria

Russell K (2003) Biology and ecology *Prunus avium*. Euforgen 6.

Schmid T (2006) *Prunus avium*. In: Enzyklopädie der Holzgewächse. pp 1–16

2.2 Endophytic bacteria in plant tissue culture: differences between easy- and difficult-to-propagate *Prunus avium* genotypes.

Quambusch M[1,3], Pirttilä AM[2], Tejesvi M V.[2], Winkelmann T[1], Bartsch M[1] (2014)

[1]Woody Plant and Propagation Physiology Section, Institute for Horticultural Production Systems, Leibniz Universitaet Hannover, Herrenhaeuser Strasse 2, 30419 Hannover, Germany; [2]Department of Biology, University of Oulu, Oulu, Finland; [3]Corresponding author (quambusch@baum.uni-hannover.de)

Type of authorship:	First author
Type of article:	Research article
Contribution to the article:	Planned and performed all experiments Analyzed the data Prepared all figures Wrote the paper
Contribution of other authors:	Anna Maria Pirttilä, Mysore V. Tejesvi, Traud Winkelmann and Melanie Bartsch contributed in design of experiments, data analysis and writing the paper
Journal:	Tree Physiology
Impact factor:	3.655
Date of publication:	May 2014
DOI:	doi:10.1093/treephys/tpu027

This publication contributed the cover image Tree Physiology Volume 34 Number 5 published in May 2014 (see 2.2.1).

Tree Physiology 34, 524–533
doi:10.1093/treephys/tpu027

Research paper

Endophytic bacteria in plant tissue culture: differences between easy- and difficult-to-propagate *Prunus avium* genotypes

Mona Quambusch[1,3], Anna Maria Pirttilä[2], Mysore V. Tejesvi[2], Traud Winkelmann[1] and Melanie Bartsch[1]

[1]Woody Plant and Propagation Physiology Section, Institute for Horticultural Production Systems, Leibniz Universitaet Hannover, Herrenhaeuser Strasse 2, 30419 Hannover, Germany; [2]Department of Biology, University of Oulu, Oulu, Finland; [3]Corresponding author (quambusch@baum.uni-hannover.de)

Received October 22, 2013; accepted March 7, 2014; published online May 7, 2014; handling Editor Ron Sederoff

The endophytic bacterial communities of six *Prunus avium* L. genotypes differing in their growth patterns during in vitro propagation were identified by culture-dependent and culture-independent methods. Five morphologically distinct isolates from tissue culture material were identified by 16S rDNA sequence analysis. To detect and analyze the uncultivable fraction of endophytic bacteria, a clone library was established from the amplified 16S rDNA of total plant extract. Bacterial diversity within the clone libraries was analyzed by amplified ribosomal rDNA restriction analysis and by sequencing a clone for each identified operational taxonomic unit. The most abundant bacterial group was *Mycobacterium* sp., which was identified in the clone libraries of all analyzed *Prunus* genotypes. Other dominant bacterial genera identified in the easy-to-propagate genotypes were *Rhodopseudomonas* sp. and *Microbacterium* sp. Thus, the community structures in the easy- and difficult-to-propagate cherry genotypes differed significantly. The bacterial genera, which were previously reported to have plant growth-promoting effects, were detected only in genotypes with high propagation success, indicating a possible positive impact of these bacteria on in vitro propagation of *P. avium*, which was proven in an inoculation experiment.

Keywords: amplified ribosomal rDNA restriction analysis, bacterial endophytes, in vitro culture, plant growth-promoting bacteria, 16S rDNA sequencing.

Introduction

Wild cherry (*Prunus avium* L.) timber is a valuable hardwood, which is used for the production of veneers and solid wood furniture. The timber is highly valued in Europe due to the reddish color and firmness of the wood, comparable to tropical trees like mahogany (Kobliha 2002). Economically most desired are fast-growing trees with straight stems (Janßen et al. 2010). To optimize growth characteristics, single plants showing a desired phenotype have been selected and are distributed under the trademark silvaSELECT®. Vegetative propagation of these certified genotypes via in vitro propagation enables high multiplication rates and stable clonal plant material (Meier-Dinkel et al. 2007). To ensure genetic diversity in forestry, a set of 31 genotypes was registered and is being propagated in vitro for tree nurseries (Janßen et al. 2010).

Under commercial conditions, severe losses are observed during in vitro rooting and acclimatization of the *P. avium* genotypes. Propagation success is strongly dependent on the genotype and shows high fluctuation over the years. These problems could not be overcome by manipulation of culture media or other growth conditions (C. Schneider, personal communication), and one possible explanation for the variation could be the presence of differing endophytic bacterial populations.

Endophytes are bacteria or fungi that, during part of their life, can live inside plant tissue without eliciting symptoms of disease (Petrini 1991). Endophytic bacteria are frequently observed in plant in vitro cultures, both in commercial laboratories and in scientific studies (Leifert et al. 1991), and often affect the in vitro propagation of trees (Ulrich et al. 2008*a*). They were previously treated mainly as contaminants that

needed to be eliminated to obtain a sterile tissue culture with healthy plant growth (Ewald et al. 1997, Leifert and Cassells 2001). A frequently used method is the addition of antibiotics to the culture medium to suppress bacterial growth (Kneifel and Leonhardt 1992, Asif et al. 2013, Bohra et al. 2013). Herman (1989) presented the idea that usually non-pathogenic or even growth-promoting bacteria can become detrimental under the special growth conditions of in vitro culture and proposed the term 'vitropaths'. In contrast, numerous studies indicate that endophytes have a positive effect on plants, for example through biosynthesis of growth-stimulating phytohormones, increase of nutrient availability and induced resistance to pathogens (Goh and Vallejos 2013). Pirttilä et al. (2004) observed that freeing buds of *Pinus sylvestris* L. of bacterial endophytes resulted in an altered morphology, which could be restored by adding endophytic products to the plant medium. The inoculation of poplar tissue cultures with a *Paenibacillus* isolate led to a higher number of and longer roots on microcuttings (Ulrich et al. 2008*a*). In a study on endophytic bacteria in strawberry tissue cultures, some isolated endophytic bacteria showed plant growth-promoting effects during the acclimatization process in the greenhouse (Dias et al. 2008).

Therefore, it was advisable to identify the bacteria in the cultured *P. avium* tissue and to use this knowledge to develop strategies to potentially influence the community structure. To our knowledge, there has been only one study of bacteria associated with *P. avium* in vitro culture, which indicated a prevalence of *Pseudomonas* sp. (Cornu and Michel 1987). Kamoun et al. (1998) reported *Pseudomonas* sp. and *Agrobacterium rhizogenes* as endophytes in the related species *Prunus cerasus* L. The first objective of this study was to analyze the bacterial population structure of tissue culture material of six different *P. avium* genotypes by both culture-dependent and culture-independent methods. Secondly, we aimed to correlate the differences between the bacterial populations of different genotypes with the propagation success in vitro. Finally, inoculation of difficult-to-propagate genotypes with bacteria isolated from easy-to-propagate genotypes was carried out in order to test the putative beneficial effects of these endophytes on rooting.

Materials and methods

Plant material and in vitro culture conditions

For the present study, six *P. avium* genotypes of the silvaSELECT® selection with varying propagation success (here defined as rooting rate × acclimatization rate, given as a percentage) were selected based on data from commercial production for the years 2009–2012 (data generously provided by C. Schneider, Institut für Pflanzenkultur, Germany). Genotypes Fama and Achilleus were difficult to propagate (marked by –), with 11.4 ± 4.9 and 9.8 ± 3.6% success, respectively. Asteria and Apollo showed high fluctuation over the years (marked ±), resulting in medium propagation success of 29.7 ± 14.1 and 23.9 ± 7.6%, respectively. Easy-to-propagate genotypes (marked +) were Neptun with 38.7 ± 6.3% and Demeter with 42.2 ± 8.6% propagation success. The propagation protocol was identical for all genotypes described here, and did not involve the use of antibiotics at any stage.

The genotypes were formerly selected at the Northwest German Forest Research Institute (NW-FVA) for their growth parameters, and in vitro cultures were established from surface-sterilized winter buds and propagated for 4–16 years via axillary shoots. The shoots were cultivated on MS medium (Murashige and Skoog 1962) containing 3% (w/v) sucrose and 0.6% Phyto Agar (Duchefa, Haarlem, The Netherlands), supplemented with 2.22 µM benzylaminopurine, 0.49 µM indole-3-butyric acid and 0.29 µM gibberellic acid-3 and adjusted to pH 5.8. Each 500-ml plastic vessel contained 80 ml of culture medium and 10 plants. The cultures were incubated at 24 °C under a 16-h photoperiod (40–56 µmol m^{-2} s^{-1}) and subcultured every 5 weeks.

Isolation of bacteria

Leaf and stem segments of in vitro shoots of each genotype were placed on nutrient agar (NA) (1% beef extract, 2% yeast extract, 5% peptone, 5% NaCl, 10% agar (w/v)) and medium 523 (Viss et al. 1991), incubated under the same conditions as the in vitro shoot cultures, and monitored for 5 weeks. Emergence of bacterial colonies was observed at the cut explant surfaces, and colonies were selected based on size, shape and color and re-streaked twice onto a fresh medium to obtain pure single colonies of each species.

DNA extraction from plant material and bacterial isolates

For extraction of total genomic DNA, 140–230 mg of single in vitro shoots was collected, frozen in liquid nitrogen and ground to a fine powder. Three shoots were harvested from each *P. avium* genotype and each plant sample contained leaf, stem and shoot tip tissue. Deoxyribonucleic acid extraction and purification was carried out using the Nucleo Spin Plant II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and the DNA was finally dissolved in 100 µl of the supplied elution buffer.

For extraction of bacterial genomic DNA, individual colonies were propagated in nutrient broth at 28 °C and 200 rpm for 1–12 days until OD_{600} reached 0.6. A pellet of 1 ml of bacterial culture was supplemented with 380 µl of extraction buffer (200 mM Tris–HCl, pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) and 10 µl of lysozyme (20 mg ml^{-1}), mixed thoroughly and incubated for 1 h at 37 °C. After adding 10 µl of proteinase K (20 mg ml^{-1}) and 20 µl of RNAse (10 mg ml^{-1}), the samples were again mixed and incubated for 15 min at 65 °C. The sample was mixed with 200 µl of 5 M potassium/3 M acetate and kept on ice

for 10 min. After centrifugation for 20 min at 13,000*g*, 500 µl of the supernatant was transferred to a fresh tube and mixed with 500 µl of isopropanol. The samples were centrifuged for 10 min and the pellet was washed twice with 75% EtOH. Finally, the DNA pellet was re-suspended in 50 µl of sterile Milli-Q water.

Polymerase chain reaction amplification and construction of 16S rDNA clone libraries

The 16S rDNA of five bacterial isolates was amplified using primers 27f (AGAGTTTGATCCTGGCTCAG) (Weisburg et al. 1991) or 799f (AACMGGATTAGATACCCKG) (Chelius and Triplett 2001) and 1492r-Y (GGYTACCTTGTTACGACTT) (Weisburg et al. 1991), as previously described (Thomas et al. 2007).

Since the total plant DNA contains mitochondrial and chloroplast 16S rDNA, primers that allow a separate amplification of bacterial rDNA were used. Primer 799f (Chelius and Triplett 2001) was originally designed to amplify bacterial sequences, exclude chloroplast DNA and give a larger mitochondrial polymerase chain reaction (PCR) product. This selectivity was confirmed in studies of other plant genera (Sun et al. 2008). Therefore, primers 799f and 1492r-Y were selected to amplify bacterial DNA from *P. avium* samples. Each 25-µl PCR contained 100 ng of template DNA, 1× reaction buffer (10 mM Tris–HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin), 100 µM dNTP, 10 pmol of each primer and 1 U of FIREPol DNA polymerase (SolisBiodyne, Tartu, Estonia). Thermal cycling conditions were: initial denaturation at 94 °C for 5 min followed by 33 cycles of 94 °C for 30 s, 52 °C for 40 s, 72 °C for 60 s and a final extension step for 5 min at 72 °C.

The PCR products of four parallel reactions were combined and electrophoretically separated, and the bacterial amplification product of ~700 bp was excised from the agarose gel. The PCR products were purified using the Nucleo Spin Gel and PCR clean-up kit (Macherey-Nagel) and cloned into vector pJet1.2 using the Clone Jet PCR cloning kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Chemically competent *Escherichia coli* DH10B were transformed with the ligation product and spread on lysogeny broth (LB) agar plates supplemented with ampicillin (100 mg l^{-1}).

Amplified ribosomal rDNA restriction analysis

To identify unique sequences for subsequent sequence analysis and to estimate the diversity of bacteria, the restriction patterns within each clone library were analyzed. Ampicillin-resistant *E. coli* colonies were picked randomly and tested by colony-PCR for correct insert length. A restriction digest with enzymes HpaII, HhaI and BsuRI was performed with 100 µg of DNA of the remaining colony-PCR sample overnight at 37 °C. Amplified ribosomal rDNA restriction analysis (ARDRA) was performed with 95 amplificates from each clone library. The restriction fragments were separated in a 2% agarose gel with 0.5× Tris–borate–EDTA buffer (80 mM Tris–borate/1 mM EDTA, pH 8.3). Restriction patterns were analyzed manually and converted into a 1–0 matrix. Bands <100 bp were not taken into account. The clones of each clone library were grouped according to restriction patterns.

Sequencing and phylogenetic analysis

One clone representing each distinct ARDRA pattern of each clone library was selected for sequencing. Plasmids containing the 16S rDNA inserts were purified using the GeneJET Plasmid Miniprep kit (Fermentas) and sequenced by Seqlab (Göttingen, Germany).

Concerning bacterial isolates, each PCR amplicon was sequenced using primers 27f and 799f to obtain a nearly full-length sequence of 16S rDNA. The presence of possible chimeric sequences was checked using a DECIPHER Find Chimeras web tool (Wright et al. 2012). All obtained sequences were compared with sequences available in the NCBI GenBank database using BLASTn (Benson and Karsch-Mizrachi 2010). The sequences of clones, isolates and reference sequences were aligned using CLC software (CLC bio, Aarhus, Denmark) and regions with gaps were removed manually. Phylogenetic trees were constructed using the neighbor-joining method of the CLC software, and statistical significance of nodes was determined by bootstrap analysis with 1000 repeats.

All nucleotide sequence data obtained in this work have been deposited in the GenBank database under the following accession numbers: KF663058–KF663062 for isolates and KF663063–KF663068 for 16S rDNA gene clones.

Inoculation with bacterial isolates

The bases of in vitro shoots of genotypes Fama and Achilleus were dipped in a suspension of 10 mM $MgSO_4$ containing the bacterial isolate (10^8 CFU ml^{-1} for *Microbacterium* sp. D-I-1, 10^4 CFU ml^{-1} for *Rhodopseudomonas* sp. N-I-2, none for control) for 5 min and transferred to rooting medium (propagation medium containing 4.9 µM indole-3-butyric acid as the sole plant growth regulator). Forty shoots were treated per variant and transferred to eight vessels (replicates), except for Fama, for which only 20 shoots were treated with N-I-2. The number of roots per shoot and the percentage of rooted plants were recorded after 3 weeks of culture under the conditions given above.

Statistical analyses

Rarefaction curves were calculated using package 'rarefaction' of program R-2.15.3 (R Development Core Team 2008) to investigate the diversity of the clone libraries. Clone coverage (Mullins et al. 1995) was calculated using the following equation:

$$\text{Coverage } (C) = (1 - n_1/N) \times 100\%,$$

where n_1 is the number of clones occurring once and N is the total number of clones examined.

Statistical differences in rooting after inoculation with bacterial isolates were determined by Dunnett's test, comparing each treatment with the control using the program R-2.15.3 (R Development Core Team 2008). Dunnett's test was based on generalized linear models for each genotype (McCullagh and Nelder 1989). Because the culture vessel was the randomized unit, the test was performed with mean values of roots per shoot or percentages of rooted shoots in each culture vessel.

Results

Culture-dependent approach

During routine propagation of *P. avium* shoots, no massive outgrowth of bacteria could be observed. The only indication for bacteria in the cultures was a very slight smear around the basal part of the cut stem of the shoot. The smear did not spread further in the medium and did not continue growing on the surface of the culture medium. By placing leaf and stem segments or crushed material from these plants on bacterial indexing media, we were able to isolate endophytes from three out of six tested *Prunus* genotypes (Figure 1). Three isolates could be identified to the species level with an identity score of 99–100% of the 16S rDNA sequence with corresponding sequences available in the NCBI database. The closest match of two isolates was only described at the genus level (Table 1). The identified bacteria differed between the *Prunus* genotypes and belonged to such diverse phylogenetic groups as Proteobacteria, Firmicutes and Actinobacteria.

Two isolates were obtained from the easy-to-propagate genotype Neptun (Table 1). Isolate N-I-2 formed white, slow-growing colonies 1 mm in diameter after 1 week and was identified as a *Rhodopseudomonas* sp. The second isolate, N-I-1, appeared within the first few days at cut edges of leaves and stems with transparent colonies. It was not possible to generate a stable pure culture, however, because growth of the bacterium ceased without plant material. Nevertheless, DNA was isolated and the strain was identified as a *Mycobacterium* sp. From the second, easy-to-propagate genotype Demeter, a bacterium with fast-growing, shiny, yellow-orange colonies was isolated and identified as *Microbacterium testaceum*. Additionally, one *Bacillus licheniformis* strain, forming first clear and later white colonies with filamentous growth, was isolated from the crushed plant material of Demeter. On samples of the difficult-to-propagate genotype Fama, bacterial colonies could only be observed after 5 weeks on medium NA or 523. The obtained isolate was identified as *Acinetobacter junii*. No bacteria could be isolated from genotypes Achilleus, Apollo and Asteria within 6 weeks of observation.

Culture-independent approach

To identify the uncultivable fraction of endophytic bacteria from the tissue cultured plants, the 16S rDNA was amplified from total plant DNA. After electrophoresis, two distinct bands of PCR products were observed. The larger fragment, deriving

Table 1. Classification of the bacterial isolates based on 16S rDNA sequences using BLASTn.

Isolate	Host plant genotype	Closest match at NCBI	Sequence length (bp)	% Identity
F-I-3	Fama (–)	*Acinetobacter junii*	1441	100
N-I-1	Neptun (+)	*Mycobacterium* sp.	706	99
N-I-2	Neptun (+)	*Rhodopseudomonas* sp.	1398	99
D-I-1	Demeter (+)	*Microbacterium testaceum*	1398	99
D-I-3	Demeter (+)	*Bacillus licheniformis*	1449	100

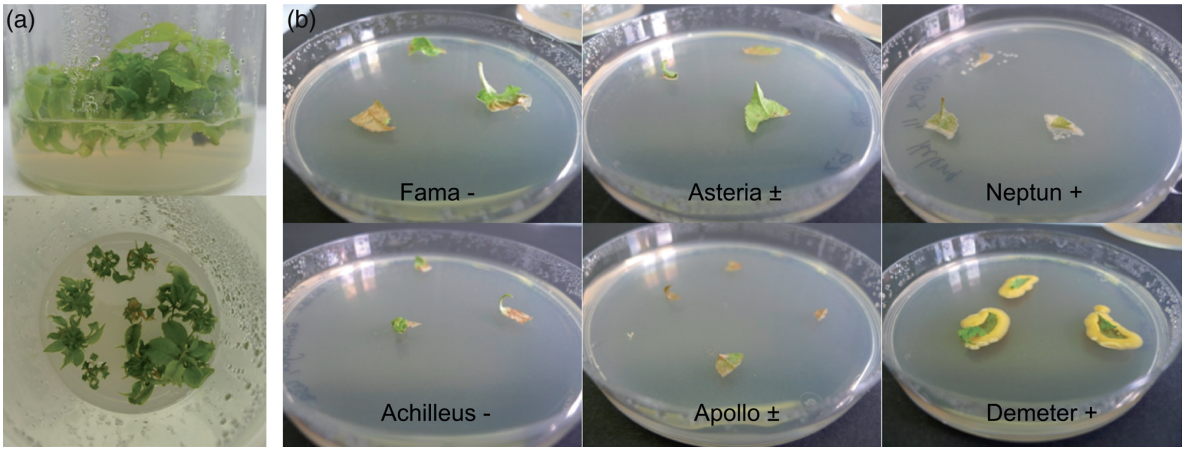


Figure 1. Bacterial isolates obtained from leaves of in vitro plant material. (a) In vitro *P. avium* shoots in the propagation phase used for the isolation of bacteria. (b) Leaf parts on bacterial indexing medium 523, 1 week after transfer. Visible growth of bacteria could only be detected in genotypes Neptun and Demeter.

from mitochondrial rDNA, was 1050 bp long and occurred in all repetitions of all *Prunus* genotypes. A smaller bacterial fragment of ~700 bp was observed in all three samples of each of the genotypes Fama, Neptun and Demeter, and in two samples of Achilleus (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). In genotypes Apollo and Asteria, the bacterial endophytes, if present, were below the detection limit with the method used. This finding correlates with the isolation experiment, where no bacterial colonies were observed in the leaves and stems of Apollo and Asteria within 6 weeks of observation. Therefore, the bacterial populations of those two genotypes were not studied further.

A purified bacterial 16S amplicon was selected for genotypes Fama, Achilleus, Neptun and Demeter and used for the construction of a 16S rDNA clone library (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). All clones were screened by ARDRA with the restriction enzymes HhaI, BsuRI and HpaII and sorted according to their restriction profiles into 9–20 operational taxonomic units (OTUs). An OTU was defined as a group of clones that had identical banding patterns obtained from digestion with the three restriction enzymes (see Figure 2 as an example for Neptun). A representative of each OTU was selected for 16S rDNA sequence analysis.

To confirm that a sample size of 95 is sufficient to cover the bacterial population of in vitro plant material, two statistical tests were used. The rarefaction analysis indicates how well taxonomic diversity is reflected by the sample size used. Because there are experimental biases (mainly DNA extraction), the rarefaction data represent the diversity within the clone library and not the bacterial population in the tissue. As the rarefaction curves of all four clone libraries nearly reached a plateau, the unique sequence types in the sample size of 95 approach the total number of sequences in the library (Figure 3). Calculation of clone coverage confirmed that >90% of the clone library diversity could be detected in all four genotypes. No chimeric sequences were found by the test software, but one clone was identified as chloroplast DNA by sequencing and therefore discarded.

Prunus genotypes contain different endophytic populations

The culture-independent method revealed differences between the endophytic populations of the four tested genotypes. Interestingly, they correlated with the propagation abilities in

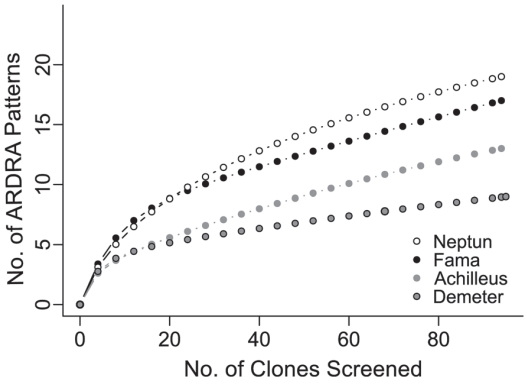
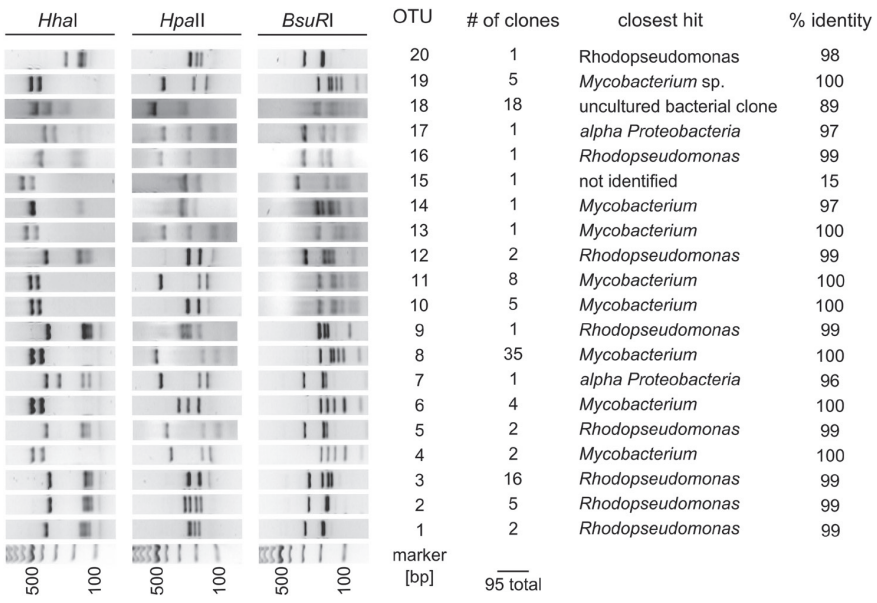


Figure 3. Rarefaction curves indicating diversity of endophytic bacterial 16S rDNA clone libraries of four *P. avium* genotypes. The frequency of different restriction profiles was plotted against the number of clones screened.



OTU	# of clones	closest hit	% identity
20	1	Rhodopseudomonas	98
19	5	*Mycobacterium* sp.	100
18	18	uncultured bacterial clone	89
17	1	*alpha Proteobacteria*	97
16	1	*Rhodopseudomonas*	99
15	1	not identified	15
14	1	*Mycobacterium*	97
13	1	*Mycobacterium*	100
12	2	*Rhodopseudomonas*	99
11	8	*Mycobacterium*	100
10	5	*Mycobacterium*	100
9	1	*Rhodopseudomonas*	99
8	35	*Mycobacterium*	100
7	1	*alpha Proteobacteria*	96
6	4	*Mycobacterium*	100
5	2	*Rhodopseudomonas*	99
4	2	*Mycobacterium*	100
3	16	*Rhodopseudomonas*	99
2	5	*Rhodopseudomonas*	99
1	2	*Rhodopseudomonas*	99
marker [bp]	95 total		

Figure 2. ARDRA banding patterns of 95 bacterial 16S rDNA fragments of a plant sample from genotype Neptun (+). One representative of each group was sequenced and compared with NCBI database entries using BLASTn.

vitro (Figure 4). The most prominent bacterial genus found in all genotypes was *Mycobacterium*. In genotypes Fama and Achilleus, both difficult to propagate, the clone library contained 95 and 87% of *Mycobacterium* spp., respectively. The easy-to-propagate genotypes both contained a second bacterial strain with high abundance (Figure 4). In Neptun we detected a *Rhodopseudomonas* sp., which accounted for 32% of the clone library. Sixty-seven percent of the clone library of the genotype Demeter contained a *Microbacterium* sp. Additionally, we found present in the clone libraries of all genotypes one OTU, which could not be identified further and showed a 99% identity to a so far uncultured Proteobacterium.

Comparing the results from the culture-independent method with the culture-dependent method, the detected bacterial populations were similar but not identical (Figure 5). *Microbacterium* sp. and *Rhodopseudomonas* sp. were detected with both methods and showed a 100% identity in the alignment of the analyzed sequences. In contrast to the culture-dependent method, the clone library represented only two phylogenetic groups, Proteobacteria and Actinobacteria. The isolates *B. licheniformis* and *A. junii* could not be detected with the culture-independent method.

Inoculation with bacterial isolates promotes rooting

As the two isolates N-I-2 (*Rhodopseudomons* sp.) and D-I-1 (*Microbacterium* sp.) were present only in the easy-to-propagate genotypes, a correlation between these bacteria and in vitro rooting ability of *P. avium* was evaluated.

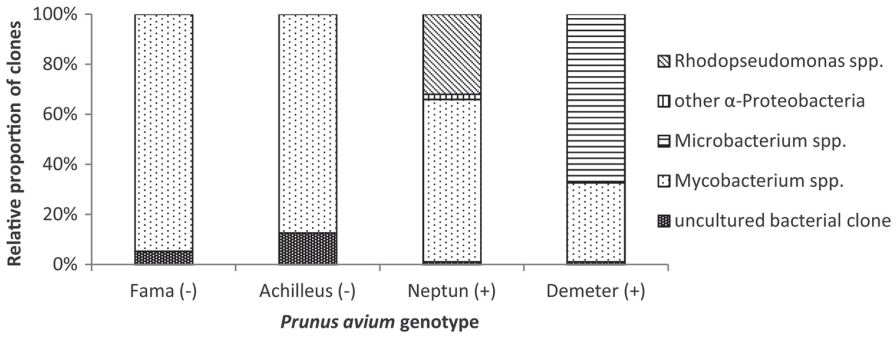


Figure 4. Composition of the bacterial community of four *Prunus* genotypes analyzed by direct amplification of bacterial 16S rDNA from in vitro plant material.

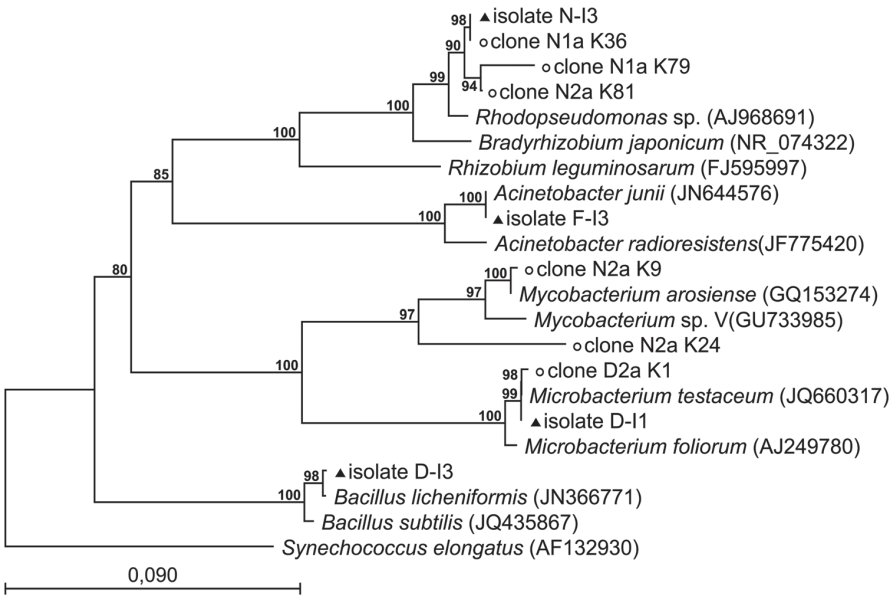


Figure 5. Phylogenetic tree based on 608 nucleotides of the 16S rDNA showing the relationship of clones (circle) and isolates (triangle) from in vitro cultures of different *P. avium* genotypes to reference sequences (the closest hit and one closely related species according to BLASTn analysis). Phylogenies were inferred using the neighbor-joining analysis. Values from 1000 bootstrap repeats are presented if support was >50%. The scale bar represents genetic differences based on the Jukes–Cantor correction. Positions containing gaps were eliminated from the dataset. Numbers in parentheses represent the sequence accession numbers in EMBL/GenBank. *Synechococcus elongatus* was used as the outgroup.

Table 2. Effect of inoculation with the bacterial isolates (see Table 1) on in vitro rooting of *P. avium* genotypes Fama and Achilleus ($n = 8$ replicates of five shoots each). Asterisks indicate significant differences between the treatments and the corresponding control by Dunnett's test (*, ** and *** indicate $P \leq 0.05$, 0.01 and 0.001, respectively).

Genotype	Treatment	Rooting (%)		Number of roots per shoot	
		Mean (±SD)	*P*-value	Mean (±SD)	*P*-value
Fama	Control	5.0 (±14.1)		0.2 (±0.5)	
	N-I-2	30.0 (±11.5)	0.0755	0.7 (±0.6)	0.3258
	D-I-1	67.5 (±23.7)	0.0003***	3.0 (±1.7)	0.0030**
Achilleus	Control	72.5 (±30.1)		4.4 (±2.3)	
	N-I-2	92.5 (±10.4)	0.142	8.3 (±2.2)	0.0014**
	D-I-1	92.5 (±14.9)	0.142	7.3 (±2.2)	0.0136*

The difficult-to-propagate genotypes Fama and Achilleus were inoculated with the two isolates. The inoculation had a positive effect on both the number of roots per shoot and the percentage of rooted plants (Table 2). Treatment with *Rhodopseudomonas* sp. N-I-2 significantly increased the number of roots per shoot of the genotype Achilleus, while *Microbacterium* sp. D-I-1 significantly stimulated both the number of roots and rooting percentage of Fama and the number of roots of Achilleus.

Discussion

To analyze the endophytic population present in *P. avium* in vitro cultures, two detection strategies employing isolation of bacterial strains on growth media (culture-dependent method) or amplification of bacterial sequences from total plant DNA (culture-independent method) were applied. Different bacterial populations were detected with the two methods used. For example, *Mycobacterium* spp. were found in high numbers in all four clone libraries, but they were isolated only once from the genotype Neptun. The occurrence of *Mycobacterium* spp. in plant tissues is often underestimated when culture-dependent methods are used, because most species of this genus are not cultivable on common bacterial growth media (Conn and Franco 2004, Koskimäki et al. 2010). On the other hand, *A. junii* and *B. licheniformis* were isolated from the plant material but could not be detected with the culture-independent method. This divergence is often seen in similar studies and shows the importance of combining both methods for a detailed view of the bacterial population (Sessitsch et al. 2002, Thomas et al. 2008, Ulrich et al. 2008*b*, Tejesvi et al. 2010). Reasons for the detection of certain species only by isolation are low abundance in the plant (only 90% of the bacterial sequences present in the clone library are detected) or difficulties in extracting their DNA from plant material.

The culture-independent method gave valuable insights on the bacterial populations in our study. The number of endophytes is usually lower in plant tissue cultures than in plants living in their natural habitat, because the culture tissue originates from only one organ, in our case winter buds, and only a subset of endophytes can survive the special conditions of in vitro culture. We were therefore able to cover >90% of the OTUs present in the clone libraries with a relatively low sample size (Figure 3) compared with studies of field samples (Chelius and Triplett 2001).

The length of the cloned colony-PCR fragments varied, which resulted in small differences in their restriction profiles and a higher number of OTUs than was obtained after sequencing and aligning the amplicons. For example, all 28 OTUs allotted to the genus *Mycobacterium* in all clone libraries were based on only two different sequences that were included in the phylogenetic tree (Figure 5). The analysis of 700 bp of the 16S rDNA with identity scores >97% allowed a reliable positioning of most OTUs at the genus level. Identity scores of 99–100% of the nearly full-length 16S rDNA sequences of isolates allowed the approximation to the species level. However, this should be further confirmed by sequencing the internal transcribed spacer (ITS) region of the strains.

The results of this study clearly show that in vitro-grown shoots of *P. avium* are associated with populations of endophytic bacteria differing in their composition between genotypes. The most dominant bacterial strain in all four *P. avium* genotypes studied (Figure 4) was a *Mycobacterium* sp. Bacteria of this genus have earlier been described as widespread contaminants in plant tissue cultures of ornamentals (Taber et al. 1991), as well as endophytes of wheat (Conn and Franco 2004), rice (Mano et al. 2007) and rock plant (Koskimäki et al. 2010). Pirttilä et al. (2005) localized a *Mycobacterium* sp. by in situ hybridization in buds of Scots pine and observed a seasonal variation with a higher number or metabolic activity of the bacteria in early spring during development of leaf primordia. Because the *Prunus* tissues in our study were derived from winter buds, it is likely that this endophyte was introduced into the tissue culture with the explants. These findings and the high abundance in our study suggest a close association of *Mycobacterium* sp. and trees. Laukkanen et al. (2000) suggested that *Mycobacterium* spp. become harmful to Scots pine cultures in vitro and may cause

growth retardation. Similarly, the observation of the present study that the difficult-to-propagate genotypes Achilleus and Fama were dominated by *Mycobacterium* spp. could indicate a detrimental effect of these bacteria on in vitro cultures. It is important to note that the diversity of *Mycobacterium* spp. in tissue cultures can be very high, as recently demonstrated for the rock plant *Pogonatherum paniceum* (P. Beauv.) Hack. (Koskimäki et al. 2010).

Besides the *Mycobacterium* sp., the two easy-to-propagate genotypes Neptun and Demeter both contained other bacteria with high abundance in the clone library, a *Rhodopseudomonas* sp. and a *Microbacterium* sp., respectively (Figure 4). This finding may suggest a positive effect of these bacterial endophytes on *Prunus* in vitro cultures, either direct or indirect, which could take place, e.g., by interaction with the *Mycobacterium* sp. Ardanov et al. (2012) have shown that the presence of one additional bacterial endophyte influences the innate endophytic community and can have varying effects on plant disease resistance. Growth-promoting effects were previously described for the detected bacterial genera: *Rhodopseudomonas* spp. are purple non-sulfur bacteria belonging to the family of Bradyrhizobiaceae that include the mutualistic *Bradyrhizobium* spp., which were placed within the same clade in the phylogenetic tree (Figure 5). A growth-promoting effect of *Rhodopseudomonas* sp. was shown in studies on tomato seedlings in vitro (Koh and Song 2007) and on greenhouse-grown plants (Lee et al. 2008), and the strains were shown to produce the plant growth hormone auxin. Auxins are especially important in the rooting phase of *Prunus* propagation (Blakesley et al. 1991). Therefore, all auxin-producing bacteria are interesting candidates for improvement of this critical cultivation step for the formation of a stable root system after acclimatization.

Microbacterium spp. have often been detected as endophytes in tissue cultures, e.g., in *Ensete ventricosum* (Welw.) Cheesman (Birmeta et al. 2004), *Carica papaya* L. (Thomas et al. 2007) and *Eleutherococcus sieboldianus* (Makino) Koidz. (Müller and Döring 2009), as well as in field samples and tissue cultures of *Robinia pseudoacacia* L. (Boine et al. 2008, Zaspel et al. 2008). The presence of *NifH*-like genes that are important for nitrogen fixation, and production of indole acetic acid and siderophores, traits that are considered beneficial for plants, have been identified in strains of the genus *Microbacterium* (Zakhia et al. 2006, Ji et al. 2014).

Bacillus and *Acinetobacter* species were commonly isolated in plant tissue and cell cultures in commercial laboratories and are often considered to be a contaminant introduced into the cultures by laboratory practice due to their high-temperature stability (Leifert et al. 1991, Isenegger et al. 2003, Donnarumma et al. 2011). On the other hand, several *Bacillus* spp. were selected as endophytes with high potential for biocontrol (Bacon and Hinton 2002).

To conclude, the endophytic populations of *P. avium* shoots are not only genotype dependent, but additionally correlate with the propagation success in vitro. While the bacterial populations of difficult-to-propagate genotypes are dominated by *Mycobacterium* spp., the easy-to-propagate genotypes both contain other potentially plant growth-promoting strains. The inoculation of genotypes Fama and Achilleus with the isolates *Rhodopseudomonas* sp. N-I-2 and *Microbacterium* D-I-1 revealed a positive effect of these bacteria on rooting of these difficult-to-propagate *P. avium* genotypes. These results need to be confirmed by additional experiments, and the mode of action will need to be further analyzed. Nevertheless, the preliminary data of the inoculation experiment support the hypothesis of pronounced effects of endophytic bacteria on the propagation ability of *P. avium* in vitro. Generally, manipulation of the endogenous bacterial population by selected culture conditions or inoculation with growth-promoting or stabilizing bacteria might improve plant survival and growth during the critical phases of plant tissue culture.

Supplementary Data

Supplementary data are available at *Tree Physiology* online.

Acknowledgments

The authors thank the Institut für Pflanzenkultur, Schnega, Germany, for providing them with the plant material and for motivating cooperative work, and Philip Pallmann, Institute of Biostatistics, Leibniz Universitaet Hannover, for support in the statistical analyses.

Conflict of interest

None declared.

Funding

This work was supported by COST Action FA1103 'Endophytes in Biotechnology and Agriculture'. Financial support of the German Federal Ministry of Economics and Technology within the program PRO INNO Grant no. KF2508004AJ0 is gratefully acknowledged. M.Q. is a member of the graduate school WeGa PhD funded by the German Federal Ministry of Education and Research and the Ministry for Science and Culture of Lower Saxony.

References

Ardanov P, Sessitsch A, Häggman H, Kozyrovska N, Pirttilä AM (2012) Methylobacterium-induced endophyte community changes correspond with protection of plants against pathogen attack. PLoS One 7:e46802.

Asif M, Eudes F, Randhawa H, Amundsen E (2013) Cefotaxime prevents microbial contamination and improves microspore embryogenesis in wheat and triticale. Plant Cell Rep 32:1637–1646.

Bacon CW, Hinton DM (2002) Endophytic and biological control potential of *Bacillus mojavensis* and related species. Biol Control 23:274–284.

Benson D, Karsch-Mizrachi I (2010) GenBank. Nucleic Acids Res 33:D34–D38.

Birmeta G, Passoth V, Roos S, Welander M (2004) Identification of bacteria and yeasts from in vitro and surface-sterilized field samples of *Ensete ventricosum* by rDNA analysis. Biotechnol Lett 26: 1867–1872.

Blakesley D, Weston GD, Hall JF (1991) The role of endogenous auxin in root initiation Part I: evidence from studies on auxin application, and analysis of endogenous levels. Plant Growth Regul 10:341–353.

Bohra P, Waman AA, Sathyanarayana BN, Umesha K, Anu SR, Swetha HG, Gourish RK (2013) Aseptic culture establishment using antibiotics with reference to their efficiency and phytotoxicity in difficult-to-establish native Ney Poovan Banana (*Musa*, AB). Proc Natl Acad Sci India Sect B Biol Sci 84:257–263.

Boine B, Naujoks G, Stauber T (2008) Investigations on influencing plant-associated bacteria in tissue cultures of black locust (*Robinia pseudoacacia* L.). Plant Cell Tissue Organ Cult 94:219–223.

Chelius MK, Triplett EW (2001) The diversity of Archaea and bacteria in association with the roots of *Zea mays* L. Microb Ecol 41:252–263.

Conn V, Franco C (2004) Endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by terminal restriction fragment length polymorphism and sequencing of 16S rRNA. Appl Environ Microbiol 70:1787–1794.

Cornu D, Michel M (1987) Bacteria contaminants in shoot cultures of *Prunus avium* L. choice and phytotoxicity of antibiotics. Acta Hortic 212:83–86.

Dias ACF, Costa FEC, Andreote FD, Lacava PT, Teixeira MA, Assumpção LC, Araújo WL, Azevedo JL, Melo IS (2008) Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. World J Microbiol Biotechnol 25:189–195.

Donnarumma F, Capuana M, Vettori C, Petrini G, Giannini R, Indorato C, Mastromei G (2011) Isolation and characterisation of bacterial colonies from seeds and in vitro cultures of *Fraxinus* spp. from Italian sites. Plant Biol 13:169–176.

Ewald D, Naujoks G, Zaspel I, Szcygiel K (1997) Occurrence and influence of endogenous bacteria in embryogenic cultures of Norway spruce. In: Cassells AC (ed.) Pathogen and microbial contamination management in micropropagation. Springer, Dordrecht, pp 149–155.

Goh C-H, Vallejos D (2013) The impact of beneficial plant-associated microbes on plant phenotypic plasticity. J Chem Ecol 39:826–839.

Herman E (1989) Non-axenic plant tissue culture: possibilities and opportunities. Acta Hortic 280:233–238.

Isenegger DA, Taylor PWJ, Mullins K, McGregor GR, Barlass M, Hutchinson JF (2003) Molecular detection of a bacterial contaminant *Bacillus pumilus* in symptomless potato plant tissue cultures. Plant Cell Rep 21:814–820.

Janßen A, Meier-Dinkel A, Steiner W, Degen B (2010) Forest genetic resources of wild cherry. Forst Holz 65:19–24.

Ji SH, Gururani MA, Chun S-C (2014) Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiol Res 169:83–98.

Kamoun R, Lepoivre P, Boxus P (1998) Evidence for the occurrence of endophytic prokaryotic contaminants in micropropagated plantlets of *Prunus cerasus* cv. 'Montmorency'. Plant Cell Tissue Organ Cult 52:57–59.

Kneifel W, Leonhardt W (1992) Testing of different antibiotics against gram-positive and gram-negative bacteria isolated from plant tissue culture. Plant Cell Tissue Organ Cult 29:139–144.

Kobliha J (2002) Wild cherry (*Prunus avium* L.) breeding program aimed at the use of this tree in the Czech forestry. J For Sci 48:202–218.

Koh R-H, Song H-G (2007) Effects of application of *Rhodopseudomonas* sp. on seed germination and growth of tomato under axenic conditions. J Microbiol Biotechnol 17:1805–1810.

Koskimäki JJ, Hankala E, Suorsa M, Nylund S, Pirttilä AM (2010) Mycobacteria are hidden endophytes in the shoots of rock plant [*Pogonatherum paniceum* (Lam.) Hack.] (Poaceae). Environ Microbiol Rep 2:619–624.

Laukkanen H, Soini H, Kontunen-Soppela S, Hohtola A, Viljanen M (2000) A mycobacterium isolated from tissue cultures of mature *Pinus sylvestris* interferes with growth of Scots pine seedlings. Tree Physiol 20:915–920.

Lee K-H, Koh R-H, Song H-G (2008) Enhancement of growth and yield of tomato by *Rhodopseudomonas* sp. under greenhouse conditions. J Microbiol 46:641–646.

Leifert C, Cassells A (2001) Microbial hazards in plant tissue and cell cultures. In Vitro Cell Dev Biol 37:133–138.

Leifert C, Ritchie J, Waites W (1991) Contaminants of plant-tissue and cell cultures. World J Microbiol Biotechnol 7:452–469.

Mano H, Tanaka F, Nakamura C, Kaga H, Morisaki H (2007) Culturable endophytic bacterial flora of the maturing leaves and roots of rice plants (*Oryza sativa*) cultivated in a paddy field. Microbes Environ 22:175–185.

McCullagh P, Nelder JA (1989) Generalized linear models. 2nd edn. Chapman & Hall/CRC, Boca Raton, FL.

Meier-Dinkel A, Steiner W, Artes O, Hosius B, Leinemann L (2007) Die silvaSELECT-Vogelkirschen- Klonmischung "Escherode I". AFZ-Der Wald 5:246–247.

Müller P, Döring M (2009) Isothermal DNA amplification facilitates the identification of a broad spectrum of bacteria, fungi and protozoa in *Eleutherococcus* sp. plant tissue cultures. Plant Cell Tissue Organ Cult 98:35–45.

Mullins TD, Britschgi TB, Krest RL, Giovannoni SJ, Britschgi B (1995) Genetic comparisons reveal the same unknown bacterial lineages in Atlantic and Pacific bacterioplankton communities. Limnol Oceanogr 40:148–158.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497.

Petrini O (1991) Fungal endophytes of tree leaves. In: Andrews JH, Hirano SS (eds) Microbial ecology of leaves. Springer, New York, pp 179–197.

Pirttilä A, Joensuu P, Pospiech H, Jalonen J, Hohtola A (2004) Bud endophytes of Scots pine produce adenine derivatives and other compounds that affect morphology and mitigate browning of callus cultures. Physiol Plant 121:305–312.

Pirttilä AM, Pospiech H, Laukkanen H, Myllylä R, Hohtola A (2005) Seasonal variations in location and population structure of endophytes in buds of Scots pine. Tree Physiol 25:289–297.

R Development Core Team (2008) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Sessitsch A, Reiter B, Pfeifer U, Wilhelm E (2002) Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and Actinomycetes-specific PCR of 16S rRNA genes. FEMS Microbiol Ecol 39:23–32.

Sun L, Qiu F, Zhang X, Dai X, Dong X, Song W (2008) Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. Microb Ecol 55:415–424.

Taber RA, Thielen MA, Falkinham JO, Smith RH (1991) *Mycobacterium scrofulaceum*: a bacterial contaminant in plant tissue culture. Plant Sci 78:231–236.

Tejesvi MV, Ruotsalainen AL, Markkola AM, Pirttilä AM (2010) Root endophytes along a primary succession gradient in northern Finland. Fungal Divers 41:125–134.

Thomas P, Kumari S, Swarna GK, Prakash DP, Dinesh MR (2007) Ubiquitous presence of fastidious endophytic bacteria in field shoots and index-negative apparently clean shoot-tip cultures of papaya. Plant Cell Rep 26:1491–1499.

Thomas P, Swarna GK, Patil P, Rawal RD (2008) Ubiquitous presence of normally non-culturable endophytic bacteria in field shoot-tips of banana and their gradual activation to quiescent cultivable form in tissue cultures. Plant Cell Tissue Organ Cult 93:39–54.

Ulrich K, Stauber T, Ewald D (2008*a*) *Paenibacillus*—a predominant endophytic bacterium colonising tissue cultures of woody plants. Plant Cell Tissue Organ Cult 93:347–351.

Ulrich K, Ulrich A, Ewald D (2008*b*) Diversity of endophytic bacterial communities in poplar grown under field conditions. FEMS Microbiol Ecol 63:169–180.

Viss P, Brooks E, Driver J (1991) A simplified method for the control of bacterial contamination in woody plant tissue culture. In Vitro Cell Dev Biol 27:42.

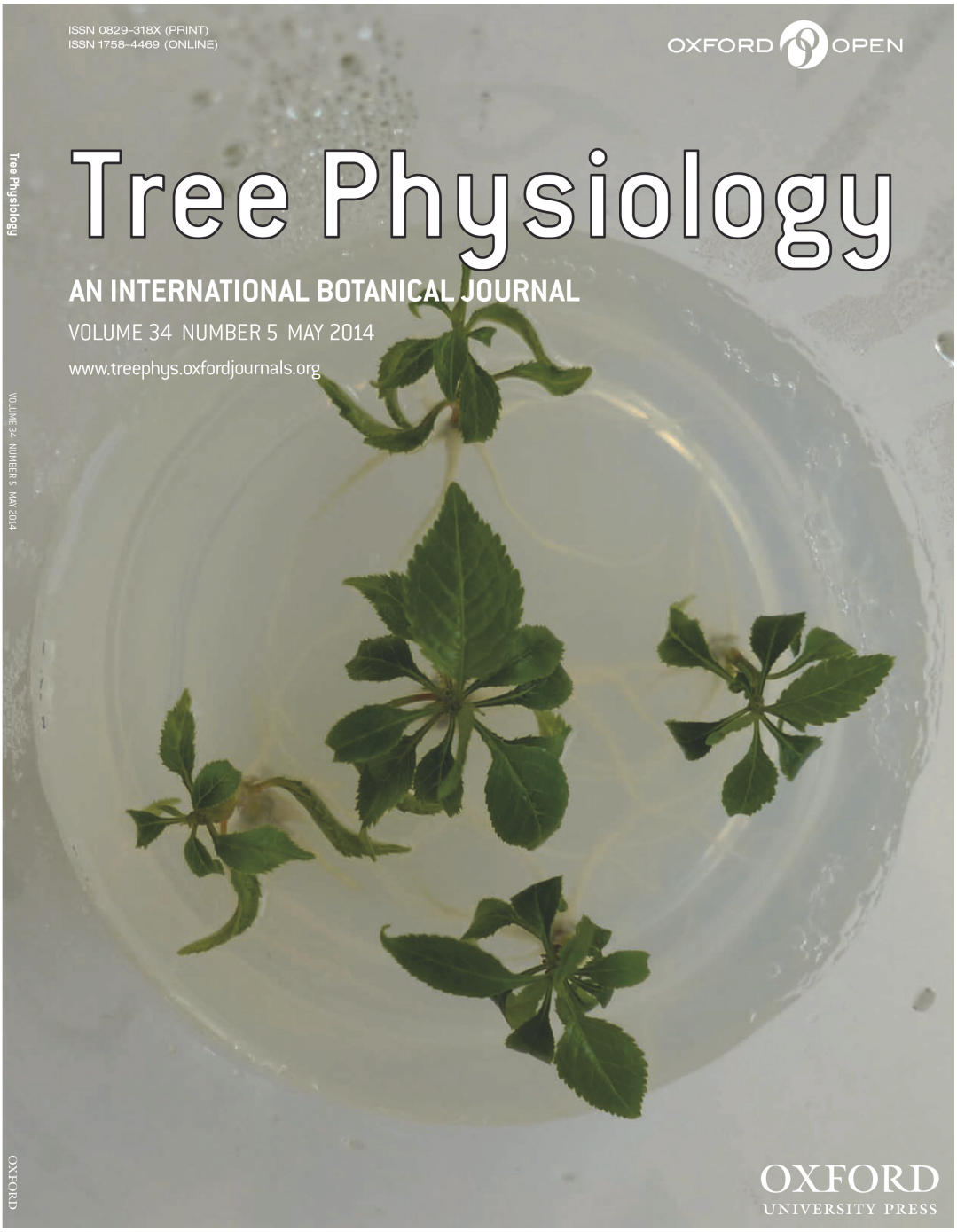
Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173: 697–703.

Wright ES, Yilmaz LS, Noguera DR (2012) DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. Appl Environ Microbiol 78:717–725.

Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B, Lajudie PDe (2006) Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for nifH-like gene within the genera *Microbacterium* and *Starkeya*. Microb Ecol 51:375–393.

Zaspel I, Ulrich A, Boine B, Stauber T (2008) Occurrence of culturable bacteria living in micropropagated black locust cultures (*Robinia pseudoacacia* L.). Eur J Hortic Sci 73:231–235.

2.2.1 Cover image Tree Physiology



Cover image: *Prunus avium* (wild cherry) plants propagated in vitro by axillary shoots and rooted on culture medium after inoculation with the endophytic bacterium isolate N-I-2 (*Rhodopseudomonas* spp.). Quambusch et al. (pages 524–533) studied the endophytic bacterial populations of different *Prunus* genotypes and found that endogenous bacteria influenced propagation success. The presence of *Rhodopseudomonas* or *Microbacterium* spp. in the plants correlated with a higher rooting percentage in vitro and the effect could be transferred to genotypes lacking these bacteria by inoculation. Photo: Mona Quambusch.

2.2.2 *Supplement*

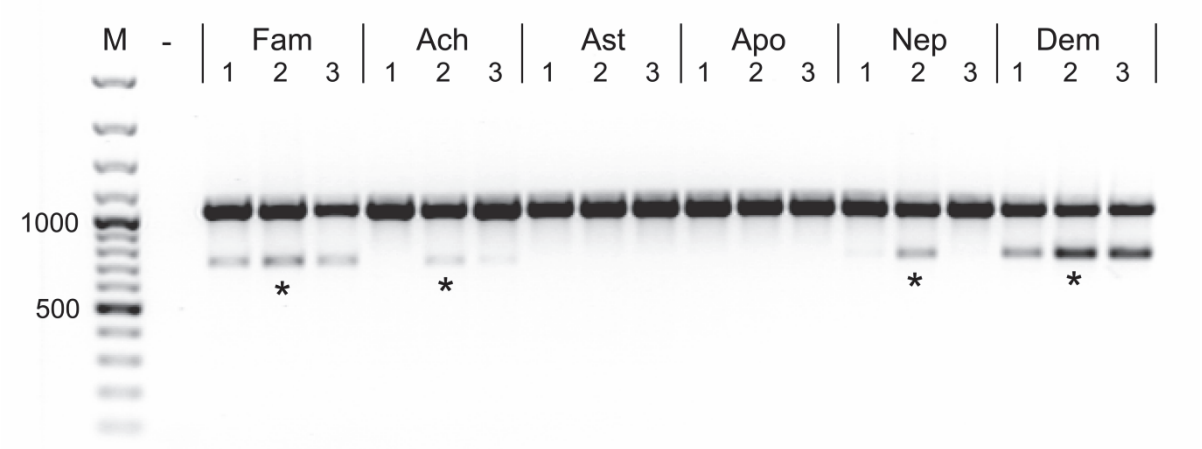


Fig. S 1 PCR on 16S rDNA of total plant DNA with primers 799f and 1492r. Three plants were tested for each genotype. The mitochondrial amplicon can be seen at 1050 bp, the amplified bacterial DNA at 700 bp. Bands marked with an asterisk were used to establish a clone library and analysed by ARDRA.

2.3 Dynamics of endophytic bacteria in plant in vitro culture – quantification of three bacterial strains in *Prunus avium* in different plant organs and in vitro culture phases.

Quambusch M, Brümmer J, Haller K, Winkelmann T, Bartsch M

Leibniz Universität Hannover, Institute of Horticultural Production Systems, Woody Plant and Propagation Physiology Section, Herrenhäuser Str. 2, 30419 Hannover, Germany, bartsch@baum.uni-hannover.de, tel: 0049 511 762 3602, fax: 0049 511 762 3608

Type of authorship:	First author
Type of article:	Research article
Contribution to the article:	Planned and performed qPCR experiments Planned and performed inoculation experiments Analyzed qPCR and inoculation data Prepared all figures Wrote the paper
Contribution of other authors:	Jane Brümmer performed and analyzed regeneration experiments as part of her bachelor thesis[3] Kristin Haller performed and analyzed auxin assays and sequenced ITS regions as part of her master thesis[4] Traud Winkelmann and Melanie Bartsch contributed to experimental design, data analysis and writing the paper
Journal:	Plant Cell Tissue Organ Culture
Impact factor:	2.125
Date of online publication:	20.04.2016
DOI:	10.1007/s11240-016-0999-0

[3] Brümmer, Jane (2014) Quantifizierung bakterialler Endophyten in *Prunus avium* In-vitro-Kulturen nach Regeneration über Adventivsprosse. Bachelor's thesis, Leibniz Universität Hannover

[4] Haller, Kristin (2014) Biochemische Charakterisierung von Bakterienisolaten aus In-vitro-Kulturen der Vogelkirsche (*Prunus avium*). Master's thesis, Leibniz Universität Hannover

Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-016-0999-0

ORIGINAL ARTICLE

Dynamics of endophytic bacteria in plant in vitro culture: quantification of three bacterial strains in *Prunus avium* in different plant organs and in vitro culture phases

Mona Quambusch[1] · Jane Brümmer[1] · Kristin Haller[1] · Traud Winkelmann[1] · Melanie Bartsch[1]

Received: 5 February 2016 / Accepted: 12 April 2016

Abstract Endophytic bacteria occurring in plant in vitro cultures have often been described as contaminants, although these are generally present in all plant tissues, often with plant growth promoting effects. The effects of bacterial endophytes in different in vitro culture phases and in different plant organs of *Prunus avium* were studied. In a previous study we investigated the endophytic bacterial community of six registered silvaSELECT® genotypes and found differences in the bacterial community that correlated with propagation success. In this study, quantitative polymerase chain reaction protocols were developed to look at the dynamics of the most abundant endophytes, *Mycobacterium* spp., *Rhodopseudomonas* spp., and *Microbacterium* spp. These endophytes were quantified during propagation and rooting, and the bacterial content in three successive years was evaluated depicting the fluctuation over time. Leaves, stems, and shoots were found to contain bacteria although in different abundance. It was shown that after regeneration via adventitious shoots the bacteria were not eliminated, but showed slightly modified concentrations. The plant growth promoting traits of the two isolates *Rhodopseudomonas palustris* N-I-2 and *Microbacterium testaceum* D-I-1 were tested in an inoculation experiment, and showed a promotion in rooting of two difficult-to-propagate *P. avium* genotypes.

Keywords Adventitious shoots · *Microbacterium* · *Rhodopseudomonas* · *Mycobacterium* · Quantitative PCR · Rooting

Abbreviations

IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ITS	16S to 23S internal transcribed spacer
NAA	1-Naphthaleneacetic acid
qPCR	Quantitative polymerase chain reaction
TDZ	Thidiazuron
WPM	Woody plant medium

Introduction

Endophytes are generally considered to be present in all plant tissues (Schulz and Boyle 2006; Hardoim et al. 2015) and their presence during in vitro culture has been increasingly under investigation (Zaspel et al. 2008; Dias et al. 2008; Almeida et al. 2009; Abreu-Tarazi et al. 2010; Donnarumma et al. 2011; Marino and Gaggìa 2015). In natural habitats, plants and endophytes are considered to be mutualists or commensals, and numerous examples for bacteria with plant growth promoting effects or biocontrol mechanisms have been described (Rosenblueth and Martínez-Romero 2006). During in vitro culture endophytes (especially bacterial endophytes) are either described as plant growth promoting or more often as detrimental. Examples of positive effects were the growth promotion of endophytic bacteria during acclimatization of strawberry (Dias et al. 2008) and pineapple tissue cultures (González-Rodríguez et al. 2013). In woody plants, inoculation of

Electronic supplementary material The online version of this article (doi:10.1007/s11240-016-0999-0) contains supplementary material, which is available to authorized users.

✉ Melanie Bartsch
bartsch@baum.uni-hannover.de

[1] Woody Plant and Propagation Physiology Section, Institute of Horticultural Production Systems, Leibniz Universität Hannover, Herrenhaeuser Str. 2, 30419 Hannover, Germany

Published online: 20 April 2016

poplar tissue cultures with a *Paenibacillus* isolate led to a higher number of and longer roots on microcuttings (Ulrich et al. 2008). Negative effects of endophytes have been described as rapid growth of bacteria on the plant tissue culture medium leading to reduced plant growth and rooting rates (Leifert and Cassells 2001). Recently, the presence of bacteria has been described to interfere with the analysis of somaclonal variation by semi-specific PCR (Moreno-Vázquez et al. 2014).

The timber of wild cherry (*Prunus avium* L.) is highly valued in Europe for its firm wood and reddish color used in furniture production and as veneer for interior finishings (Kobliha 2002; Schmid 2006). Trees with growth characteristics, such as fast growth and straight bole have been selected and distributed under the trademark silvaSELECT® (Janßen et al. 2010). Vegetative propagation of these certified genotypes via in vitro culture enables high multiplication rates and stable clonal plant material to be produced (Meier-Dinkel et al. 2007). In commercial production, however, severe losses were observed during in vitro rooting and acclimatization. The propagation success was strongly dependent on the genotype and showed a high fluctuation over the years that could, to date, not be overcome by improved growth medium or other growth conditions (Schneider, personal communication).

We have investigated the endophytic bacterial community of six registered silvaSELECT® genotypes with differing propagation success (Quambusch et al. 2014). By culture-independent analysis, a bacterial spectrum with low diversity consisting of three dominating bacterial genera was observed. The most abundant bacterial group was *Mycobacterium* spp., which was identified in the clone libraries of all analyzed *Prunus* genotypes. Only in the easy-to-propagate genotypes 'Neptun' and 'Demeter', bacteria belonging to the genera *Rhodopseudomonas* and *Microbacterium* were detected as the second most abundant bacteria, which were previously reported to have plant growth-promoting effects (Koh and Song 2007; Ji et al. 2014). The results of a preliminary inoculation experiment indicated a positive impact of the corresponding isolates N-I-2 (*Rhodopseudomonas* sp.) and D-I-1 (*Microbacterium* sp.) on in vitro propagation of the difficult-to-propagate *P. avium* genotypes 'Fama' and 'Achilleus' (Quambusch et al. 2014).

Three quantitative polymerase chain reaction (qPCR) protocols were developed to look at the dynamics of *Mycobacterium* spp., *Rhodopseudomonas* spp. and *Microbacterium* spp. during different phases of in vitro culture of six *Prunus* genotypes. Quantitative PCR is a versatile technique for the accurate, sensitive, and high-throughput detection and quantification of bacteria in planta mostly targeting the 16S ribosomal DNA (Ruppel et al. 2006; Andreote et al. 2009). The first objective was to quantify the bacteria in different plant organs (leaf, stem, and roots) during in vitro propagation in three successive years. The second objective was to test whether shoots derived from a single or few cells by adventitious shoot regeneration might contain different amounts of the endophytes compared to the explant donor material. Preliminary results indicated promoting effects of bacteria during rooting (Quambusch et al. 2014), which were verified in three independent inoculation experiments using *Rhodopseudomonas* sp. strain N-I-2 and *Microbacterium* sp. strain D-I-1 that had been isolated from easy-to-propagate genotypes.

Materials and methods

Plant material

Six *P. avium* genotypes of the silvaSELECT® selection were used. Genotypes 'Fama' and 'Achilleus' were difficult-to-propagate, 'Asteria' and 'Apollo' showed high fluctuation over the years, resulting in medium propagation success (defined as rooting rate × acclimatization rate), and 'Neptun' and 'Demeter' were easy-to-propagate (Quambusch et al. 2014). The shoot cultures were originally established between 1997 and 2009 (Supplementary material 1) in vitro from winter buds (Meier-Dinkel 1986) and obtained from the Institut fuer Pflanzenkultur (Schnega, Germany) in July 2011.

The shoots were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3 % (w/v) sucrose, 0.6 % Phyto Agar (Duchefa, Haarlem, The Netherlands), 2.2 μM benzylaminopurine, 0.5 μM indole-3-butyric acid (IBA), and 0.3 μM gibberellic acid-3 and the pH adjusted to 5.8 (Quambusch et al. 2014). Each 500 ml plastic vessel contained 80 ml culture medium and 10 shoots. The cultures were incubated at 24 °C under a 16-h photoperiod (33–61 μmol/m^2/s) and subcultured to fresh medium every 5 weeks. The same conditions were used for the 3 weeks rooting-phase, except that 4.9 μM IBA was used as the sole plant growth regulator and makronutrients were reduced to 1/3 MS.

The plant material for qPCR was harvested after 5 weeks of culture. Plant material for rooted shoots was harvested 3 weeks after transfer to rooting medium. For comparison between organs the youngest five to seven leaves, the stem, and the roots of rooted shoots were harvested from the same plant, frozen, and ground in liquid nitrogen to a fine powder. Stem and leaf material during propagation was harvested in February 2013, and stem, leaf, and root material during rooting was harvested in November 2012. For the comparison of bacterial content over time, the samples were harvested in September 2011, February 2013, and November 2013. Although the number

of subcultures and the month of sampling was not equal between these harvesting dates these are referred to as year 1, 2, and 3 (after cultivation in our lab). Each sample contained material of the whole shoot, including leaf, stem, and shoot tip. In addition, leaf, stem and root samples at the end of the rooting phase were analyzed. An overview of all sample types and sampling times is provided in Supplementary material 1. The DNA extraction protocol was described previously (Quambusch et al. 2014) with the amount of plant material used for genomic DNA extraction reduced to 50–100 mg. For each variant three independent samples were harvested and processed if not stated differently. The concentration of extracted DNA was adjusted to 10 ng/µl prior to qPCR analysis.

Bacterial strains

The bacterial strains analyzed had been identified by culture-independent and -dependent detection methods in *P. avium* in vitro cultures (Quambusch et al. 2014). The *Microbacterium* sp. strain D-I-1 was isolated from genotype 'Demeter', *Rhodopseudomonas* sp. strain N-I-2 was isolated from genotype 'Neptun', and *Mycobacterium* spp. were detected in all analyzed genotypes, but could not be isolated on the bacterial culture medium used. The species abbreviation "sp." is used where only one specific species, often an isolated strain, is addressed, and the abbreviation "spp." where all species of one genus were referred to. *Mycobacterium* sp. strain F-I-6 was isolated from genotype 'Fama' and cultivated on Middlebrook 7H10 medium (Middlebrook and Cohn 1958), a medium specially developed for the isolation and cultivation of mycobacteria.

The protocol for the extraction of DNA from bacterial isolates, amplification, and sequencing of the 16S rDNA was described previously (Quambusch et al. 2014). *Mycobacterium* strain F-I-6 was classified by 16S rDNA sequencing at the genus level (Table 1). In order to allocate the isolates to species in the respective genera, all three isolates were further classified by sequencing of the 16S to 23S internal transcribed spacer (ITS) region using the primers FGPS 1490-72 (TGCGGCTGGATCCCCTCCTT) and FGPL 132-38 (CCGGGTTTCCCCATTCGG) (Koskimäki et al. 2010). Thermal cycling conditions were: initial denaturation at 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s, and a final extension step for 5 min at 72 °C. The isolates are closest related to *Microbacterium testaceum*, *Rhodopseudomonas palustris,* and *Mycobacterium colombiense* (Table 1).

GenBank accession numbers of 16S rDNA sequence of the strains are KF663058.1 for *M. testaceum* strain D-I-1, KF663061.1 for *R. palustris* strain N-I-2, and KF663062.1 for *M. colombiense* strain N-I-1. New nucleotide sequence data obtained in this work have been deposited in the GenBank database under the following accession numbers: KU852575 for 16S rDNA of *Mycobacterium* sp. strain F-I-6 and KU852572–KU852574 for ITS sequences of the three isolates.

Quantitative PCR

The extracted gDNA was used to quantify 16S rDNA of *Mycobacterium* spp., *Microbacterium* spp., and *Rhodopseudomonas* spp. in the plant material. Specific primers for the bacterial genera were developed based on alignments of the 16S rDNA of all bacteria detected by cultivation-dependent and -independent methods in the plant material in previous work (Quambusch et al. 2014) (Table 2). A test with the software Probe Match of Ribosomal Database Project (Cole et al. 2014) confirmed specificity at the genus level.

The specificity of the obtained primers was validated and verified with genomic bacterial DNA and total plant DNA. Each 25-µl PCR contained 50 ng template DNA, 1× reaction buffer (10 mM Tris–HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.001 % gelatin (Platin, extrapure, Carl Roth, Karlsruhe, Germany), 100 µM dNTP, 10 pmol of each primer and 1 U FIREPol DNA polymerase (SolisBiodyne, Tartu, Estonia). Thermal cycling conditions were: initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 61 °C for 30 s, 72 °C for 15 s, and a final extension step for 5 min at 72 °C.

To optimize qPCR conditions, the primers were tested at 59.5, 61, and 62.5 °C. The 61 °C gave the best ratio of efficiency and specificity and was chosen for further analysis. The effect of template amount used for the qPCR was linear to the amount of detected 16S rDNA in the

Table 1 Classification of the bacterial isolates based on 16S rDNA and ITS sequences using BLASTn

Isolate	Closest match at NCBI	Region	Sequence length (bp)	% Identity
D-I-1	*Microbacterium testaceum*	ITS	562	87.76
N-I-2	*Rhodopseudomonas palustris*	ITS	1018	80.00
F-I-6	*Mycobacterium colombiense*	ITS	437	96.87
F-I-6	*Mycobacterium colombiense*	16S	1382	99.93

The 16S rDNA data for isolates D-I-1 and N-I-2 were previously published (Quambusch et al. 2014)

Table 2 List of primers developed for the quantification of *Rhodopseudomonas* spp., *Microbacterium* spp., and *Mycobacterium* spp.

Primer name	Sequence (5′ → 3′)	Amplicon size (bp)
Rhod 51-70 f	CCGTTCGTGGGTTTACTCAC	217
Rhod 244-268 r	TAGGCTCCGAAGAGAAGGCT	
Micro 60-79 f	CATTCCACGGTTTCCGTGAC	182
Micro 221-242 r	CATTTCTGGCCCGTTCTGGTG	
Myco 52-72 f	GTGGGTTTCCTTCCTTGGGAT	217
Myco 246-269 r	ACACAGGCCACAAGGGAAC	

range of 5–50 ng and therefore 10 ng was used for all further experiments. The resulting qPCR conditions were as follows: Each 15 µl reaction contained 10 ng template DNA, 1× iTaq Universal SYBR^{TM} Green Supermix (Bio-Rad, München, Germany), and 500 nM of each primer. Thermal cycling conditions were: initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing/extension at 61 °C for 30 s. A melt-curve analysis was added from 65 to 93.5 °C with 0.5 increment for 5 s each. A standard curve was generated for each qPCR run with a dilution series of gDNA of *Microbacterium* sp. D-I-1 (1 ng, 100, 10, 1 pg, 100 and 10 fg), gDNA of *Rhodopseudomonas* sp. N-I-2 (1 ng, 100, 10, 1 pg, 100, 10 and 1 fg) or purified plasmid DNA containing one copy of 16S rDNA of *Mycobacterium* sp. (100, 10, 1 pg, 100, 10, 1 and 0.1 fg). For *Mycobacterium* sp. the plasmid was used because no sufficient amount of gDNA was available. Each variant was analyzed with three biological replicates each measured in technical duplicates with the mean value of the technical replicates used for further calculations. The amount of bacteria in each sample was calculated from the standard curve constructed by plotting the mean Ct values for each DNA dilution against the initial quantity of template DNA using the Bio-Rad CFX Manager 3.1 Software.

One amplification product of each primer set was purified using the Nucleo Spin Gel and PCR clean-up kit (Macherey–Nagel, Düren, Germany) and sequenced by Seqlab (Göttingen, Germany). The obtained sequences were aligned with the bacterial 16S rDNA using the CLC software (CLC bio, Aarhus, Denmark).

The detection limit was tested by a standard curve with 10 ng plant material as background. For *Microbacterium* D-I-1 and *Rhodopseudomonas* N-I-2, 1 fg to 10 ng of isolated bacterial gDNA were added in steps of ten. As no bacterial gDNA was available for *Mycobacterium* sp., 0.1 fg to 1 ng of plasmid containing *Mycobacterium* 16S rDNA was used. Total DNA extracted from in vitro propagated *P. avium* 'Rube' was used as background and as negative control in all experiments. This genotype did not show endophytic bacterial growth in vitro, but could contain latent bacteria in non-cultivable stage.

Adventitious shoot formation

Shoot cultures of the six genotypes were used as source material for adventitious shoot formation. To induce regeneration of adventitious shoots, leaves and internode sections were transferred to two different media: regeneration medium 1 was based on woody plant medium (WPM) (Lloyd and McCown 1980) supplemented with 4.5 µM thidiazuron (TDZ), 1.6 µM 1-naphthaleneacetic acid (NAA), and 0.8 % (w/v) Plant Agar (Duchefa, Haarlem, The Netherlands); regeneration medium 2 was based on half strength WPM and half strength Driver and Kuniyuki walnut medium (Driver and Kuniyuki 1984) containing 9.1 µM TDZ, 2.5 µM IBA (Matt and Jehle 2005), and 0.37 % (w/v) Gelrite (Duchefa, Haarlem, The Netherlands). The youngest fully developed leaves were used and slit slightly three times diagonal to the midrib prior to transfer to the regeneration medium. For internode explants, all axillary meristematic tissue was carefully removed and the final length was 1–3 mm. In total, 713 leaf and 386 internode explants were prepared (for detailed information on replicate numbers see Supplementary material 2). Five explants were plated on one culture vessel (9 cm Petri dish). The explants were cultivated for 8 weeks with a transfer to fresh medium after 4 weeks under the conditions described previously for the shoot cultures. The developing adventitious shoots were separated from the explants and cultivated as previously described for further growth for one to two culture passages. Shoots were always kept separate based on their origin. For qPCR analysis, samples of the explant donor material were taken from standard propagated plants at the time of transfer to regeneration medium (for details see Supplementary material 1). Samples from adventitious shoots were taken after one or two subcultures to reach a sufficient amount of material. Only internode samples were harvested from the explant donor material and compared to the corresponding regenerated shoots, and the same procedure was applied for leaf explants. Photos were taken using a stereomicroscope (Stemi 2000-C, Zeiss, Göttingen, Germany) using the camera Axio Cam MRc and the AxioVs40V 4.7.2. software (Zeiss, Göttingen, Germany).

Inoculation with bacterial isolates

The difficult-to-propagate genotypes 'Fama' and 'Achilleus' were inoculated with the bacterial strains N-I-2 (*R. palustris*) and D-I-I (*M. testaceum*) isolated from the easy-to-propagate genotypes 'Neptun' and 'Demeter', respectively. The inoculation protocol was described by Quambusch et al. (2014). Forty shoots were treated per variant and transferred to eight 500 ml vessels (replicates) filled with 80 ml medium. The experiment was repeated four times (01/2014, 07/2014, 01/2015 and 12/2015), preliminary data of the first experiment were previously published (Quambusch et al. 2014). Because of contamination the final number of vessels varied from 23 to 31 in total for each variant. The number of roots per shoot (including unrooted shoots) and the percentage of rooted shoots were recorded after 3 weeks of culture. Three inoculated plants and one control plant from experiment one were randomly sampled, surface desinfected (5 min treatment with 2 % sodium hypochlorite followed by three rinses for 2-, 3-, and 5-min in sterile water), and the DNA was extracted to test the spread of the inoculated bacterial strains in the plant material by qPCR. The last rinse fluid was plated on bacterial growth medium to confirm the effectiveness of the surface desinfection.

Statistical analysis

All statistics were performed with the program R-3.2.2 (R Development Core Team 2008). Statistical differences in the amount of DNA between organs and years were determined by pairwise comparison of means using Tukey test based on linear mixed-effects model for comparison of different organs and linear model for comparison between years. An analysis of variance (ANOVA) (Type II Wald Chi square test) was conducted to describe variation among and between groups. The bacterial content of adventitious shoots and its source material within one genotype were compared using the Welch Two Sample *T* test and level of significance of $\alpha = 5$ %. Dependence between propagation success and concentration of *Mycobacterium* spp. was calculated using Pearson's correlation coefficient (r). Data of the inoculation experiment were tested by three-way ANOVA (genotype × treatment × experiment repetition) and differences between control and treatment were calculated using Dunnett's test based on generalized linear models for each genotype.

Results

qPCR establishment

To confirm the quantification of the three bacterial strains, one bacterial amplification product for each primer set was sequenced and compared to 16S rDNA entries of the NCBI GenBank. All three qPCR products could be verified in comparison to the original isolates *Microbacterium* sp. D-I-1 (accession number KF663058.1), *Rhodopseudomonas* sp. N-I-2 (KF663061.1) and *Mycobacterium* sp. N-I-1 (KF663062.1). The specificity of the primers was additionally verified by PCR with total plant DNA of all six *P. avium* genotypes and genomic bacterial DNA of all strains originally isolated from the plant material including *Microbacterium* sp. D-I-1, *Rhodopseudomonas* sp. N-I-2, *Acinetobacter* sp., *Micrococcus* sp., *Variovorax* sp., *Pseudomonas* sp., *Kocuria* sp., two *Bacillus* spp. and plasmid DNA of *Mycobacterium* sp. N-I-1. Amplification products were only present with the corresponding template of each primer set (data not shown). The quantification results are described on genus level, because the presence of non-cultivable bacteria of the same genus cannot be entirely excluded. The detection limit was defined as the first template amount clearly distinguishable from no template control (NTC) and negative plant sample (for details see Supplementary material 3). The sensitivity for the detection of *Mycobacterium* was very high with 0.1 fg in 10 ng total DNA which was equivalent to 30 plasmids containing one copy of 16S rDNA. As a result of the amplification of background material in the negative plant sample of up to 90 fg (from plant DNA or other bacteria) the detection limit for *Microbacterium* spp. was identified as 100 fg. For *Rhodopseudomonas* spp. the detection limit was defined as 1 fg. Primer dimers were only present in samples with no or very low template DNA in all primer combinations, but together with the melt curve analysis a clear separation of primer dimers (melting temperature 79 °C) and amplification product (melting temperature 87 °C) was possible (see Supplementary material 3). Samples showing primer dimers were treated as below detection limit.

Quantification of bacterial endophytes in different plant organs

Mycobacterium spp. were present in all plant organs of all genotypes both during in vitro propagation and the rooting phase (Fig. 1). *Rhodopseudomonas* and *Microbacterium* spp. were only found in 'Neptun' and 'Demeter' from which they were originally isolated. *Microbacterium* and *Rhodopseudomonas* were therefore only analyzed in 'Demeter' and 'Neptun', respectively, in subsequent qPCRs. When comparing stem and leaf tissue of *P. avium* in the propagation phase, the stem had a higher DNA content of *Mycobacterium* spp. (Fig. 1a–c). While this difference was only significant for 'Asteria' and 'Demeter' (Fig. 1a) a significant difference between organs was found in all genotypes (ANOVA; $p = 8.7 \times 10^{-8}$). *Microbacterium* spp. were found in higher prevalence in the leaf than in the stem in

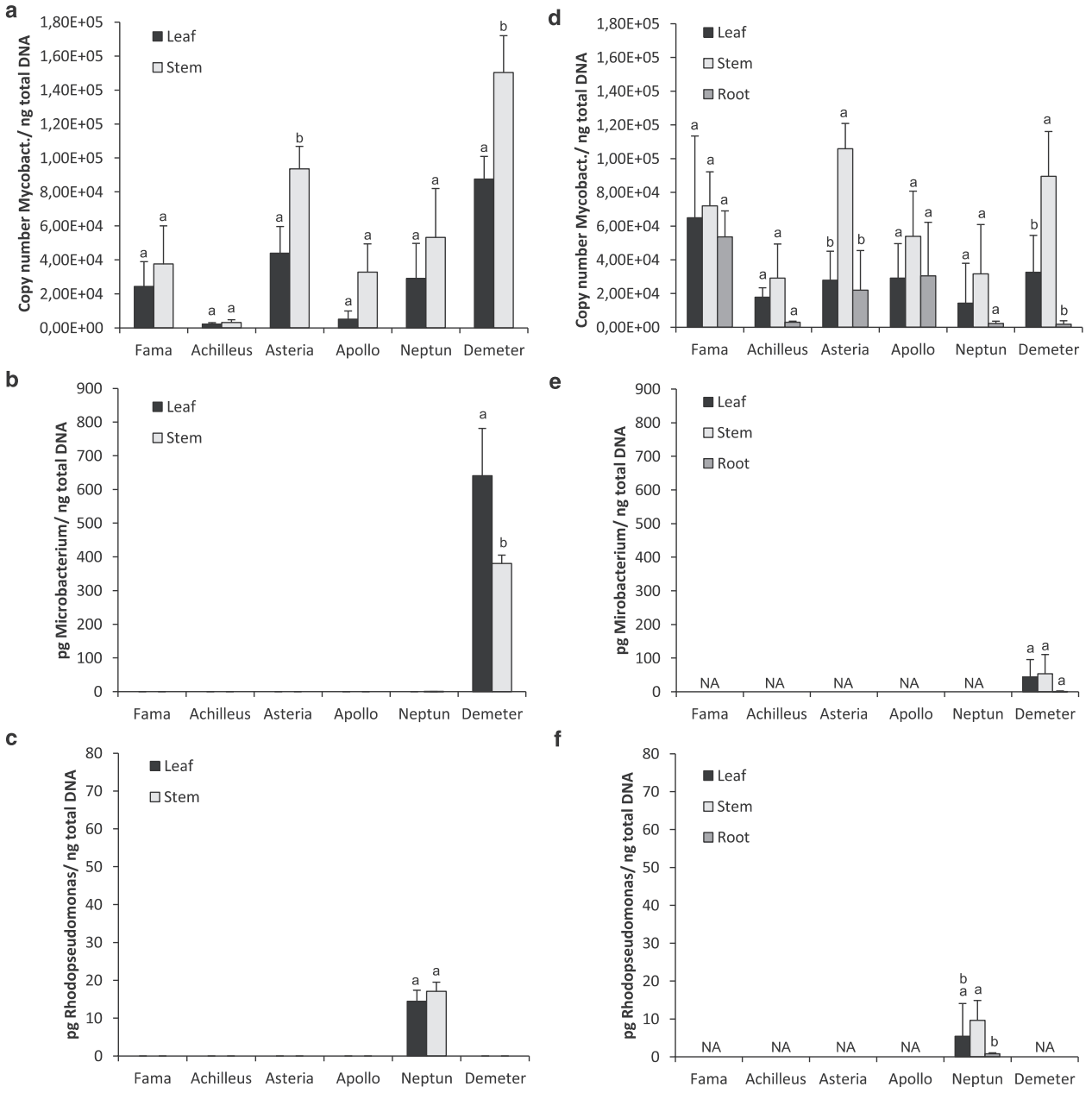


Fig. 1 Comparison of bacterial DNA content in different organs of *P. avium* microcuttings by quantitative PCR. **a–c** Propagation phase, **d–f** rooting phase. **a**, **d** 16S rDNA copy number of *Mycobacterium* spp. **b**, **e** pg gDNA of *Microbacterium* spp. **c**, **f** pg gDNA of *Rhodopseudomonas* spp. *Different letters* indicate significant differences of means in pairwise comparison between organs of one genotype (Tukey test; $p < 0.05$), *error bar* = SD, n = 3, *NA* not analyzed

'Demeter' (Fig. 1b), whereas DNA of *Rhodopseudomonas* spp. was spread equally over both stem and leaf tissue of 'Neptun' (Fig. 1c). The same effects could be seen during the rooting phase with 'Asteria' and 'Demeter' again showing significantly higher amounts of *Mycobacterium* spp. in the stems compared to the leaves and roots, and a highly significant effect ($p = 1.2 \times 10^{-7}$) of the plant organ on this bacterium calculated by ANOVA (Fig. 1d). The amount of *Microbacterium* gDNA was not significantly different over the plant organs in 'Demeter' (Fig. 1e) . *Rhodopseudomonas* gDNA was significantly less abundant in the root than in the stem of 'Neptun' (Fig. 1f). Over all genotypes and all bacteria the tendency of a lower amount of bacterial DNA could be seen in the root than in the rest of the plant.

Variation of endophytes over cultivation time

Comparing the development of the content of endophytic *Mycobacterium* spp. during cultivation time there was a significant difference between the 3 years (ANOVA; $p = 0.005$). A strong fluctuation of endophyte concentrations was visible for most genotypes (Fig. 2). The highest fluctuation was measured in 'Achilleus' with 1.7×10^4 ($\pm 1.7 \times 10^4$) copies of 16S rDNA per ng total DNA in year 1, 4.7×10^3 ($\pm 1.8 \times 10^3$) in year 2, and 1.2×10^5 ($\pm 2.1 \times 10^4$) in year 3. The fluctuation in 'Fama' was comparatively low with 7.2×10^4 ($\pm 3.2 \times 10^4$), 7.1×10^4 ($\pm 9.4 \times 10^3$), and 4.0×10^4 ($\pm 9.3 \times 10^3$) copies of *Mycobacterium* 16S rDNA in year 1, 2, and 3, respectively.

The content of *Microbacterium* spp. in 'Demeter' was more stable and quantified as 293.9 (±159.4) pg bacterial DNA per ng total DNA in year 1, 279.6 (±36.1) in year 2, and 180.1 (±121.6) in year 3. Genotype 'Neptun' contained 10.2 (±5.3), 27.5 (±9.1), and 9.6 (±7.5) pg DNA of *Rhodopseudomonas* spp. per ng total DNA in year 1, 2, and 3, respectively. As can be seen in the standard deviation within the groups the bacterial content of all three endophytes was also highly variable between single plants.

To analyze whether the abundance of bacteria correlated with the propagation success in vitro, the propagation rates were recorded in year 1 and 3 and plotted against the DNA amount of *Mycobacterium* spp. With $r = -0.44$ ($r^2 = 0.15$) and $p = 0.083$ the correlation between the two variables was not significant.

Quantification of endophytes in adventitious shoots

Formation of callus was visible on leaf explants after 1 week and on internode explants 2 weeks after transfer to the regeneration media (Fig. 3a, b). The callus formed near the cut edge of the midrib, at the cut end of the petiole, or at the cut ends of the internodes, and showed various colors from white over green and brown to red. Four weeks after transfer to regeneration medium 1 100 % (except for 'Achilleus') of leaf and stem explants showed callus formation. In addition to callus, the direct formation of globular bodies was observed for all genotypes (Fig. 3a). On regeneration medium 2 the amount of callus was lower in 'Neptun', 'Asteria', and 'Achilleus' with 30, 87, and 75 % after 4 weeks, respectively.

Adventitious shoots were formed 3 weeks after transfer to regeneration medium for internode explants and 4 weeks after transfer for leaf explants (Fig. 3c, d) and occurred directly from globular bodies and indirectly from callus. After transfer to propagation medium, bacteria were visible adjacent to some adventitious shoots, for 'Demeter' notably yellow colonies resembling colonies of *Microbacterium* sp. isolate

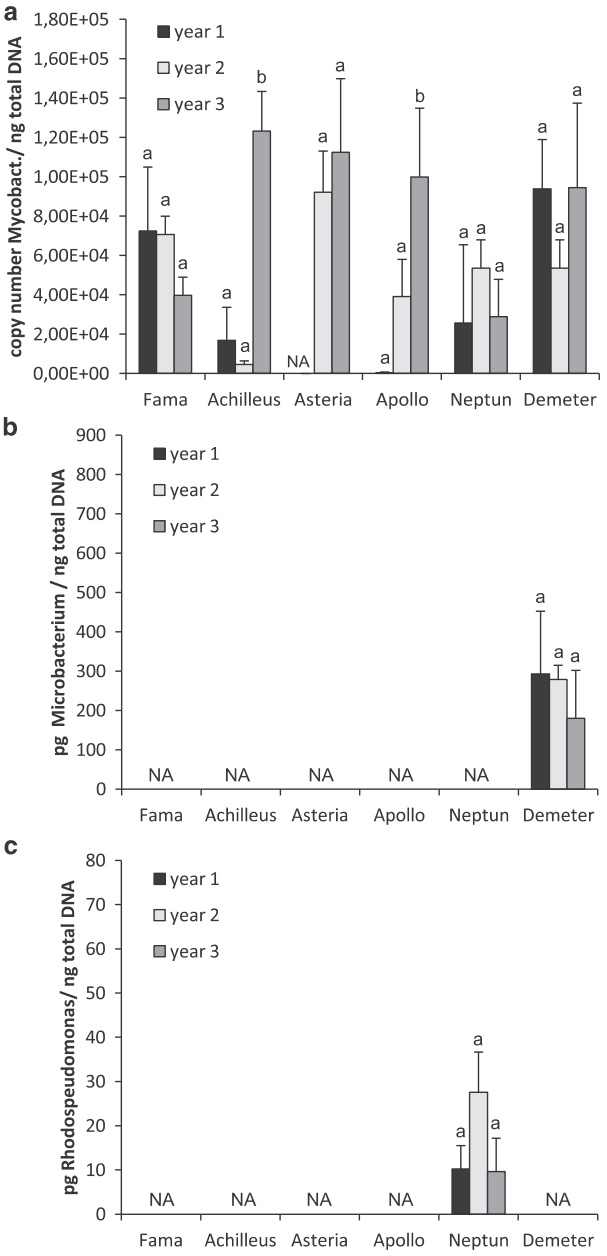


Fig. 2 Comparison of the bacterial DNA content in *P. avium* microcuttings over 3 years of propagation by quantitative PCR. **a** 16S rDNA copy number of *Mycobacterium* spp. **b** pg gDNA of *Microbacterium* spp. **c** pg gDNA of *Rhodopseudomonas* spp. *Different letters* indicate significant differences of means in pairwise comparison between organs of one genotype (Tukey test; $p < 0.05$, *error bar* = SD, n = 3). The amount of *Microbacterium* spp. and *Rhodopseudomonas* spp. was only evaluated in 'Demeter' and 'Neptun', respectively, as the first qPCR showed very low or no DNA content (Fig. 1). *NA* not analyzed

D-I-1 in appearance. Only a small number of adventitious shoots could be successfully cultured on propagation medium to obtain total plant DNA for qPCR analysis of the endophytic bacteria (71 adventitious shoots regenerated from 1099

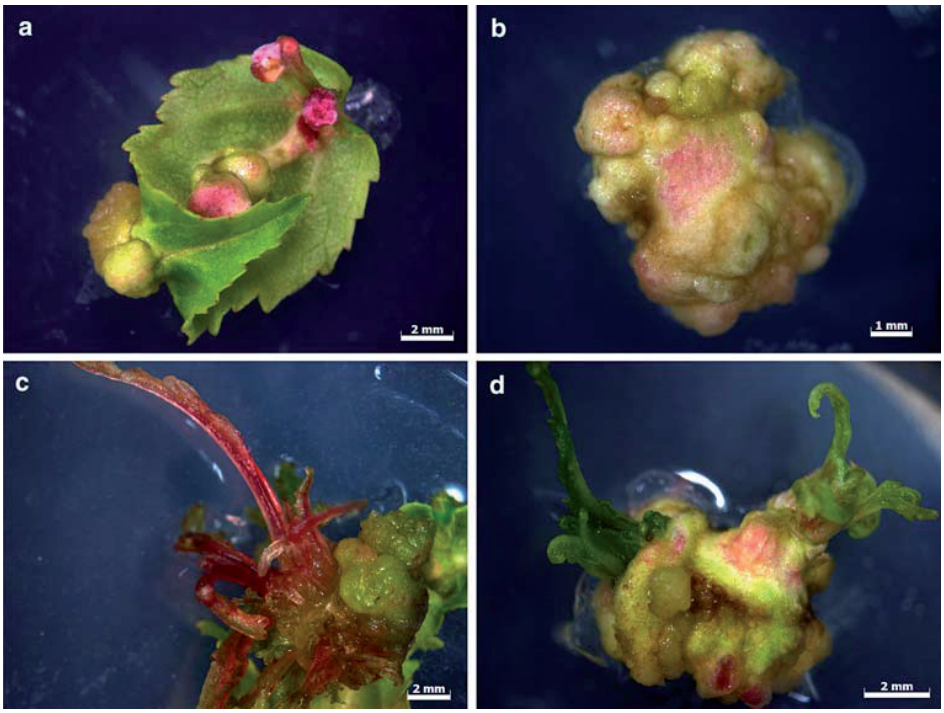


Fig. 3 Development of adventitious shoots of genotype 'Demeter'. **a** Leaf explant 2 weeks and **b** internode explant 3 weeks after transfer to regeneration medium; **c** leaf explant after 5 weeks and **d** internode explant after 4 weeks showing small adventitious shoots. Medium = regeneration medium 1

explants, 23 of which led to stable in vitro cultures, for details see Supplementary material 2).

Endophytic bacteria were not eliminated from the plant material after regeneration via adventitious shoots. For *Mycobacterium* spp. a tendency of a reduced amount of bacterial gDNA was observed in all *Prunus* genotypes, being significant only for 'Demeter' with 6.7×10^4 ($\pm 1.9 \times 10^4$) in the source material compared to 2.9×10^4 ($\pm 8 \times 10^3$) copies per ng total DNA in adventitious shoots (Fig. 4a). In single samples of adventitious shoots of 'Neptun' and 'Achilleus' the rDNA of *Mycobacterium* spp. was just above the detection limit with 280 and 88 copies per ng total DNA. *Rhodopseudomonas* spp. showed an increase from 37.4 (±1.6) pg/ng total DNA in the source material to 67.8 (±1.5) pg/ng total DNA in the adventitious shoot of 'Neptun' (Fig. 4b) (two adventitious shoots from the same explant were measured). No significant difference was detected for *Microbacterium* spp. in 'Demeter' (Fig. 4c) between the explant donor material and the regenerated shoots.

Inoculation with *Rhodopseudomonas* and *Microbacterium*

Quantification of the bacterial strains in a small random set of three inoculated plants in one inoculation experiment confirmed the presence of the bacteria in the plant tissue: 218 (±313) fg in samples inoculated with N-I-2; 285 (±189) fg in samples inoculated with D-I-1. We observed a high variability of the data between the four experiments and between the replicates (culture vessels). Nevertheless, the growth promoting effect of the inoculation was measurable. *Rhodopseudomonas palustris* strain N-I-2 led to significantly more roots in 'Achilleus' with 6.4 (±2.8) roots per shoot compared to 4.4 (±1.9) roots in control shoots (Fig. 5a). Inoculation with *Microbacterium testaceum* strain D-I-1 led to a significant increase of both, the number of roots per shoot and the percentage of rooted shoots in both genotypes. The strongest effect was measured with 'Achilleus' with 6.5 (±2.7) roots per shoot and 85.2 (±20.2) % of rooted shoots compared to 70.3 (±25.2) % in the control (Fig. 5a, b).

Discussion

Most studies on endophytes concentrate on the identification and taxonomic classification of bacterial populations at the family or even phylum level (Shakya et al. 2013; Cardinale et al. 2015). Changes in the bacterial community were mostly analyzed by denaturing gradient gel electrophoresis or pyrosequencing methods (Marques et al. 2014; Cardinale et al. 2015) giving an important insight on community shifts, but having a high bias as a result of the

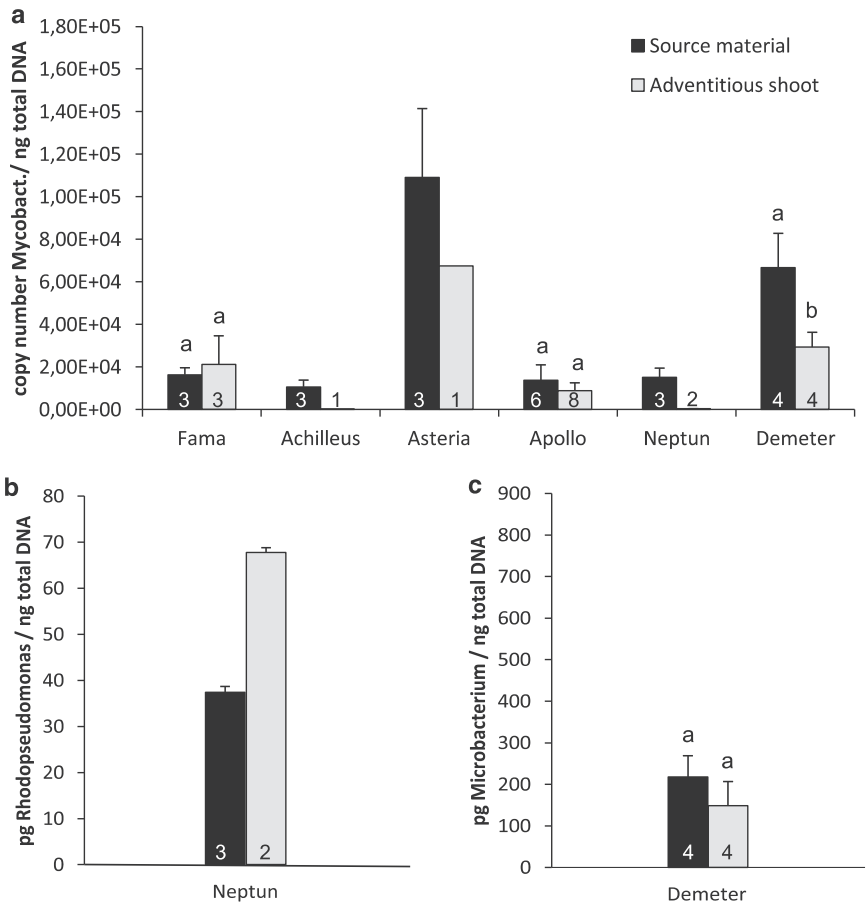


Fig. 4 Comparison of the bacterial content in *P. avium* microcuttings from standard propagation material and regenerated adventitious shoots by quantitative PCR. **a** 16S rDNA copy number of *Mycobacterium* spp. **b** pg gDNA of *Microbacterium* spp. **c** pg of gDNA of *Rhodopseudomonas* spp. *Different letters* indicate significant differences of source and adventitious shoot material within one genotype. No statistical analysis was performed if n ≤ 2. *Error bar* = SD, n values are given in the *bars*

indirect work with operational taxonomic units (OTUs) of short 16S rDNA sequences (Ghyselinck et al. 2013).

While qPCR was commonly used to monitor bacteria in planta, to our knowledge this is the first detailed analysis of the dynamics of endophytes in plant in vitro cultures including the direct quantification of all dominant strains of the bacterial population. Previous studies include this method to analyze endophytes, e.g. to monitor a single plant growth promoting strain after inoculation of the plant (Lacava et al. 2006; Ruppel et al. 2006; Peralta et al. 2012; Faleiro et al. 2013) or to quantify changes in the bacterial community in response to pathogen infection (Trivedi et al. 2010).

The bacterial population was previously described for 'Fama', 'Achilleus', 'Neptun', and 'Demeter', and the bacteria analyzed by qPCR covered 87–99 % of the detected bacterial population (Quambusch et al. 2014). This was possible as a result of the unique situation of a strongly reduced bacterial community presumably resulting from the long duration of culture of the *P. avium* genotypes under standardized conditions with previous surface desinfection common for in vitro propagation. After sequencing of a larger fragment of the 16S rDNA and additionally the ITS region (Table 1), the three most abundant bacteria could be assigned to the species *Mycobacterium colombiense*, *Rhodopseudomonas palustris,* and *Microbacterium testaceum*.

One outcome of the comparison of the abundance of the three endophytic bacteria in different plant organs was the spread over the entire plant material. Neither *Mycobacterium* spp. nor *Microbacterium* spp. or *Rhodopseudomonas* spp. were limited to only one plant organ (Fig. 1). Systemic spread of endophytes over all plant tissues was seen for many bacteria of different origin and taxonomic classification (Quadt-Hallmann et al. 1997; Compant et al. 2005).

The amount of bacteria in *P. avium* shoots was found to be highly variable over time, even under the standardized conditions present during in vitro culture propagation. This fluctuation was most evident in the comparison of the bacterial DNA amount in planta over 3 years where for example the *Mycobacterium* spp. in 'Achilleus' increased 25-fold from year 2 to year 3 of propagation in our lab (Fig. 2a). This shift in the concentration was obvious between experiments where each experiment had a different sampling time (see Figs. 1a–f, and 2 in comparison). It

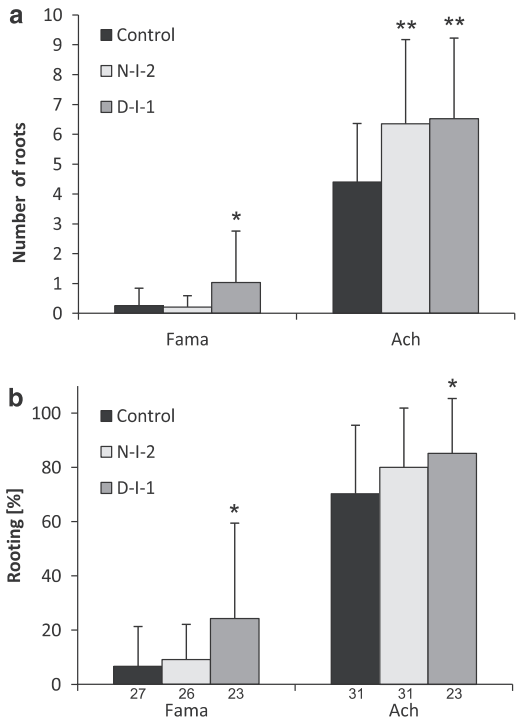


Fig. 5 Effect of inoculation with the bacterial isolates N-I-2 (*R. palustris*) and D-I-1 (*M. testaceum*) on **a** number of roots and **b** percentage of rooted plants of *P. avium* genotypes 'Fama' and 'Achilleus'. *Asterisks* indicate significant differences between the treatments and the corresponding control by Dunnett's test (* and ** indicate $p \leq 0.05$ and $p \leq 0.01$, respectively); n values are given below the *bars*

was known from other studies on tree species that the endophyte community during in vitro culture propagation was dependent on age and origin of the plant culture and the physiological status (Boine et al. 2008).

The high abundance in all plant organs without any symptoms suggested a close and non-pathogenic interaction between *P. avium* and *Mycobacterium* spp. during in vitro culture. In contrast Laukkanen et al. (2000) suggested that *Mycobacterium* spp. may cause growth retardation in Scots pine in vitro cultures. Bacteria of the genus *Mycobacterium* were regularly detected in plant tissue culture (Taber et al. 1991; Pirttilä et al. 2005; Koskimäki et al. 2010), but no growth promoting effect was described. Whether the interaction is of mutualistic or commensal nature, and whether or how the plant benefits from the bacterial colonization with *Mycobacterium* spp. remains to be investigated.

For the other two analyzed genera, growth promoting effects have previously been described. *Rhodopseudomonas* spp. belongs to the order Rhizobiales and was shown to produce indole-3-acetic acid (IAA) and increase root and shoot length in a study on tomato seedlings (Koh and Song 2007). Different species of the genus *Microbacterium* were regularly found as endophytes in in vitro cultures, for example in banana (Thomas et al. 2008) and *Robinia pseudoacacia* (Boine et al. 2008), can produce IAA (Tsavkelova et al. 2005), and contain NifH-like genes (Zakhia et al. 2006; Ji et al. 2014). In the analyzed *P. avium* genotypes these two bacteria were only detected and isolated from the two easy-to-propagate genotypes 'Neptun' and 'Demeter'. The results of the inoculation of the two difficult-to-propagate genotypes 'Fama' and 'Achilleus' with the isolates *M. testaceum* D-I-1 and *R. palustris* N-I-2 add the improvement of the rooting of in vitro shoots (see Fig. 5) to the previously described growth promoting traits. Based on the detailed analysis of four repetitions we could statistically underlay the effect of the inoculation on in vitro rooting already described in a preliminary experiment (Quambusch et al. 2014), despite the differences in rooting efficiency between the experiments and the genotypes, which was a prerequisite for the reliable classification of the isolates within the plant growth promoting bacteria.

Because rooting is essential for the acclimatization of shoot cultures and high losses were observed in commercial production of *P. avium* in this phase, even small optimization of the percentage of rooted plants or the root number are of importance. The presence of the isolates within the plant tissue 3 weeks after inoculation could be shown by qPCR. If the inoculation leads to a stable community within the plant, it could be tested, if inoculation at earlier times during propagation would be favourable to extend the positive effects of the endophytes to the whole phase of root induction.

In a classical microbiological characterization the two isolates D-I-1 and N-I-2 did not show strong growth promoting traits (data not shown). The auxin production ability was analyzed in a spectrophotometric assay based on Salkowski reagent (Rahman et al. 2010) and showed the production of a low amount (27 µg/ml) of auxin only for isolate D-I-1 (*Microbacterium* sp.) after cultivation in liquid medium supplemented with tryptophan (data not shown). The phosphate solubilization on Pikovskaya agar (Nautiyal 1999) was also low for D-I-1 and negative for N-I-2 and both isolates tested negative for siderophore production (Louden et al. 2011) (data not shown). These findings, in addition to the successful growth promotion in the inoculation assay, showed the low informative value of classical screening assays for evaluating bacterial strains for use in in vitro culture. In this artificial surrounding, a sufficient amount of auxin and all required nutrients is supplied by the culture medium. Therefore, indirect effects on the plant growth performance by shifts in the endophytic community were more likely and retaining or restoring the balance within the bacterial diversity was of

importance. To further clarify the role of the different bacterial and plant interaction partners, a colocalization microscopy in planta, for example by fluorescence in situ hybridization microscopy or the transformation of the bacteria with fluorophores would be interesting approaches.

The standard procedure to eliminate specific pathogens from in vitro culture material is meristem culture (Van den Houwe et al. 1998; Laimer da Mâchado et al. 2001). However, Pirttilä et al. (2000, 2005) have illustrated by 16S rRNA in situ hybridization that endophytes, including a *Mycobacterium* sp., were abundant in the buds of Scots pine, and could be found in the meristematic tissue with high abundance in scale primordia. Therefore, in a new approach, the influence of adventitious shoot formation on the bacterial community was tested. Regeneration of adventitious shoots occurs after cells from organized tissues dedifferentiate and then differentiate again and reorganize to form a new meristem either directly or indirectly with an intermediate callus stage (Tang et al. 2002). In contrast to our hypothesis the analyzed endophytic bacteria were not significantly reduced in abundance by the regeneration step (see Fig. 4). *Rhodopseudomonas* spp. even showed a higher abundance in the adventitious shoots than in the source material. This result supports the observation that all tested plant organs were colonized by the three bacterial strains. It is important to note that the amount of samples used for qPCR was low and can only give a tendency in the quantitative comparison between source material and adventitious shoot material. The low number of adventitious shoots was a result of a low regeneration rate and high genotypic variability typical for regeneration of *P. avium* (Yang and Schmidt 1992; Matt and Jehle 2005). Because of the high sensitivity of the qPCR we can conclude that none of the adventitious shoots measured was completely free of the previously abundant bacterial strain. Whether it is possible to generate endophyte-free material in rare occasions cannot be answered here.

An in situ hybridization study of Pirttilä et al. (2005) has shown high metabolic activity of endophytes including a *Mycobacterium* sp. before elongation and during differentiation in contrast to nearly no 16S rRNA detection in fully developed tissues. Eliminating endophytes from bud explants by various antibiotics resulted in altered tissue morphology, and the original tissue morphology could be restored by adding endophytic products to the plant growth medium (Pirttilä et al. 2004). Also during regeneration of adventitious shoots of *P. avium*, in situ hybridizations would allow the localization at the single cell level and the correlation of the abundance of certain bacteria with growth and viability of the regenerating shoots.

In conclusion, we showed qPCR to be a powerful tool to quantify endophytic bacteria in in vitro cultures to study the dynamics and interactions with the host plant. Fluctuations in the endophytic bacterial composition as was observed might contribute to instabilities and low reproducibilities of tissue culture protocols. Further insight into the proportions and relationships of different endophytic bacteria and the effect on regeneration, propagation, and rooting are needed to enable novel ways of improving in vitro propagation systems.

Acknowledgments The authors thank the Institut für Pflanzenkultur, Schnega, Germany, for providing the plant material and for motivating cooperative work, and our technicians Bärbel Ernst, Ewa Schneider and Friederike Schröder for their support. Financial support of the German Federal Ministry of Economics and Technology within the program PRO INNO Grant No. KF2508004AJ0 is gratefully acknowledged. M. Q. is a member of the graduate school WeGa Ph.D. funded by the German Federal Ministry of Education and Research and the Ministry for Science and Culture of Lower Saxony.

Author contributions Conceived and designed the experiments: MQ, TW, MB. Performed the experiments: MQ, JB, KH. Analyzed the data: MQ, JB, KH, TW, MB. Wrote the paper: MQ, TW, MB.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Abreu-Tarazi MF, Navarrete AA, Andreote FD, Almeida CV, Tsai SM, Almeida M (2010) Endophytic bacteria in long-term in vitro cultivated "axenic" pineapple microplants revealed by PCR-DGGE. World J Microbiol Biotechnol 26:555–560. doi:10.1007/s11274-009-0191-3

Almeida CV, Andreote FD, Yara R, Tanaka FAO, Azevedo JL, Almeida M (2009) Bacteriosomes in axenic plants: endophytes as stable endosymbionts. World J Microbiol Biotechnol 25:1757–1764. doi:10.1007/s11274-009-0073-8

Andreote F, Azevedo J, Araújo W (2009) Assessing the diversity of bacterial communities associated with plants. Braz J Microbiol 40:417–432. doi:10.1590/S1517-83822009000300001

Boine B, Naujoks G, Stauber T (2008) Investigations on influencing plant-associated bacteria in tissue cultures of black locust (*Robinia pseudoacacia* L.). Plant Cell Tissue Organ Cult 94:219–223. doi:10.1007/s11240-008-9395-8

Cardinale M, Grube M, Erlacher A, Quehenberger J, Berg G (2015) Bacterial networks and co-occurrence relationships in the lettuce root microbiota. Environ Microbiol 17:239–252. doi:10.1111/1462-2920.12686

Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM (2014) Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res 42:D633–D642. doi:10.1093/nar/gkt1244

Compant S, Reiter B, Sessitsch A, Clément C, Barka EA, Nowak J (2005) Endophytic colonization of *Vitis vinifera* L. by plant

growth-promoting bacterium Burkholderia sp. strain PsJN. Appl Environ Microbiol 71:1685–1693. doi:10.1128/AEM.71.4.1685

Dias ACF, Costa FEC, Andreote FD, Lacava PT, Teixeira MA, Assumpção LC, Araújo WL, Azevedo JL, Melo IS (2008) Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. World J Microbiol Biotechnol 25:189–195. doi:10.1007/s11274-008-9878-0

Donnarumma F, Capuana M, Vettori C, Petrini G, Giannini R, Indorato C, Mastromei G (2011) Isolation and characterisation of bacterial colonies from seeds and in vitro cultures of *Fraxinus* spp. from Italian sites. Plant Biol 13:169–176. doi:10.1111/j.1438-8677.2010.00334.x

Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut root stock. Hort Sci 19:507–509

Faleiro AC, Pereira TP, Espindula E, Cristiano F, Brod A, Brod FCA, Arisi ACM (2013) Real time PCR detection targeting nifA gene of plant growth promoting bacteria *Azospirillum brasilense* strain FP2 in maize roots. Symbiosis 61:125–133. doi:10.1007/s13199-013-0262-y

Ghyselinck J, Pfeiffer S, Heylen K, Sessitsch A, De Vos P (2013) The effect of primer choice and short read sequences on the outcome of 16S rRNA gene based diversity studies. PLoS One 8:e71360. doi:10.1371/journal.pone.0071360

González-Rodríguez RM, Serrato R, Molina J, Aragón CE, Olalde V, Pulido LE, Dibut B, Lorenzo JC (2013) Biochemical and physiological changes produced by *Azotobacter chroococcum* (INIFAT5 strain) on pineapple in vitro-plantlets during acclimatization. Acta Physiol Plant 35:3483–3487. doi:10.1007/s11738-013-1373-z

Hardoim PR, Van Overbeek LS, Berg G, Pirttilä M, Compant S, Campisano A, Döring M (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol Mol Biol Rev 79:293–320. doi:10.1128/MMBR.00050-14

Janßen A, Meier-Dinkel A, Steiner W, Degen B (2010) Forstgenetische Ressourcen der Vogel-Kirsche/Forest genetic resources of wild cherry. Forst und Holz 65:19–24

Ji SH, Gururani MA, Chun S-C (2014) Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. Microbiol Res 169:83–98. doi:10.1016/j.micres.2013.06.003

Kobliha J (2002) Wild cherry (*Prunus avium* L.) breeding program aimed at the use of this tree in the Czech forestry. J For Sci 48:202–218

Koh R-H, Song H-G (2007) Effects of application of *Rhodopseudomonas* sp. on seed germination and growth of tomato under axenic conditions. J Microbiol Biotechnol 17:1805–1810

Koskimäki JJ, Hankala E, Suorsa M, Nylund S, Pirttilä AM (2010) Mycobacteria are hidden endophytes in the shoots of rock plant [*Pogonatherum paniceum* (Lam.) Hack.] (Poaceae). Environ Microbiol Rep 2:619–624. doi:10.1111/j.1758-2229.2010.00197.x

Lacava PT, Li WB, Arau WL, Azevedo JL, Hartung JS (2006) Rapid, specific and quantitative assays for the detection of the endophytic bacterium *Methylobacterium mesophilicum* in plants. J Microbiol Methods 65:535–541. doi:10.1016/j.mimet.2005.09.015

Laimer da Mâchado M, Heinrich M, Hanzer V, Arthofer W, Strommer S, Paltrinieri S, Martini M, Bertaccini A, Kummert J, Davies D (2001) Improved detection of viruses and phytoplasmas in fruit tree tissue cultures. Acta Hortic 550(463–470):2001. doi:10.17660/ActaHortic.550.70

Laukkanen H, Soini H, Kontunen-Soppela S, Hohtola A, Viljanen M (2000) A mycobacterium isolated from tissue cultures of mature *Pinus sylvestris* interferes with growth of Scots pine seedlings. Tree Physiol 20:915–920. doi:10.1093/treephys/20.13.915

Leifert C, Cassells A (2001) Microbial hazards in plant tissue and cell cultures. In Vitro Cell Dev Biol Plant 37:133–138. doi:10.1079/IVP2000129

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Comb Proc Int Plant Prop Soc 30:421–427

Louden BC, Lynne AM, Haarmann D (2011) Use of blue agar CAS assay for siderophore detection. J Microbiol Biol Educ 12:51–53. doi:10.1128/jmbe.v12i1.249

Marino BG, Gaggìa F (2015) Antimicrobial activity of *Melia azedarach* fruit extracts for control of bacteria in inoculated in vitro shoots of "MRS 2/5" plum hybrid and calla lily and extract influence on the shoot cultures. Eur J Plant Pathol 141:505–521. doi:10.1007/s10658-014-0559-6

Marques JM, da Silva TF, Vollu RE, Blank AF, Ding G-C, Seldin L, Smalla K (2014) Plant age and genotype affect the bacterial community composition in the tuber rhizosphere of field-grown sweet potato plants. FEMS Microbiol Ecol 88:424–435. doi:10.1111/1574-6941.12313

Matt A, Jehle JA (2005) In vitro plant regeneration from leaves and internode sections of sweet cherry cultivars (*Prunus avium* L.). Plant Cell Rep 24:468–476. doi:10.1007/s00299-005-0964-6

Meier-Dinkel A (1986) In vitro Vermehrung ausgewählter Genotypen der Vogelkirsche (*Prunus avium* L.). Allg Forst-u J-Ztg 157:139–144

Meier-Dinkel A, Steiner W, Artes O, Hosius B, Leinemann L (2007) Die silvaSELECT-Vogelkirschen- Klonmischung „Escherode I". AFZ-Der Wald 5:246–247

Middlebrook G, Cohn ML (1958) Bacteriology of tuberculosis: laboratory methods. Am J Public Health Nations Health 48:844–853

Moreno-Vázquez S, Larrañaga N, Uberhuaga EC, Braga EJB, Pérez-Ruíz C (2014) Bacterial contamination of in vitro plant cultures: confounding effects on somaclonal variation and detection of contamination in plant tissues. Plant Cell Tissue Organ Cult. doi:10.1007/s11240-014-0553-x

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270. doi:10.1016/S0378-1097(98)00555-2

Peralta KD, Araya T, Valenzuela S, Sossa K, Martínez M, Peña-Cortés H, Sanfuentes E (2012) Production of phytohormones, siderophores and population fluctuation of two root-promoting rhizobacteria in *Eucalyptus globulus* cuttings. World J Microbiol Biotechnol 28:2003–2014. doi:10.1007/s11274-012-1003-8

Pirttilä AM, Laukkanen H, Pospiech H, Myllylä R, Hohtola A (2000) Detection of intracellular bacteria in the buds of Scotch pine (*Pinus sylvestris* L.) by in situ hybridization. Appl Environ Microbiol 66:3073–3077. doi:10.1128/AEM.66.7.3073-3077.2000.Updated

Pirttilä AM, Joensuu P, Pospiech H, Jalonen J, Hohtola A, Pirttilä A, Joensuu P, Pospiech H, Jalonen J, Hohtola A (2004) Bud endophytes of Scots pine produce adenine derivatives and other compounds that affect morphology and mitigate browning of callus cultures. Physiol Plant 121:305–312. doi:10.1111/j.1399-3054.2004.00330.x

Pirttilä AM, Pospiech H, Laukkanen H, Myllylä R, Hohtola A (2005) Seasonal variations in location and population structure of endophytes in buds of Scots pine. Tree Physiol 25:289–297

Quadt-Hallmann A, Hallmann J, Kloepper JW (1997) Bacterial endophytes in cotton: location and interaction with other plant-associated bacteria. Can J Microbiol 43:254–259

Quambusch M, Pirttilä AM, Tejesvi MV, Winkelmann T, Bartsch M (2014) Endophytic bacteria in plant tissue culture: differences between easy- and difficult-to-propagate *Prunus avium* genotypes. Tree Physiol 34:524–533. doi:10.1093/treephys/tpu027

Rahman A, Sitepu IR, Tang S-Y, Hashidoko Y (2010) Salkowski's reagent test as a primary screening index for functionalities of rhizobacteria isolated from wild dipterocarp saplings growing naturally on medium-strongly acidic tropical peat soil. Biosci Biotechnol Biochem 74:2202–2208. doi:10.1271/bbb.100360

R Development Core Team (2008) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna

Rosenblueth M, Martínez-Romero E (2006) Bacterial endophytes and their interactions with hosts. Mol Plant Microbe Interact 19:827–837. doi:10.1094/MPMI-19-0827

Ruppel S, Rühlmann J, Merbach W (2006) Quantification and localization of bacteria in plant tissues using quantitative real-time PCR and online emission fingerprinting. Plant Soil 286:21–35. doi:10.1007/s11104-006-9023-5

Schmid T (2006) *Prunus avium*. In: Roloff A, Weisgerber H, Lang U, Stimm B (eds) Enzyklopädie der Holzgewächse. Wiley, Weinheim, pp 1–16

Schulz BJE, Boyle CJC, Sieber TN (eds) (2006) What are endophytes? In: Microbial root endophytes. Springer, Berlin, pp 1–13

Shakya M, Gottel N, Castro H, Yang ZK, Gunter L, Labbe J, Muchero W, Bonito G, Vilgalys R, Tuskan G, Podar M, Schadt CW, Labbé J, Muchero W, Bonito G, Vilgalys R, Tuskan G, Podar M, Schadt CW (2013) A multifactor analysis of fungal and bacterial community structure in the root microbiome of mature *Populus deltoides* trees. PLoS One 8:e76382. doi:10.1371/journal.pone.0076382

Taber RA, Thielen MA, Falkinham JO, Smith RH (1991) *Mycobacterium scrofulaceum*: a bacterial contaminant in plant tissue culture. Plant Sci 78:231–236. doi:10.1016/0168-9452(91)90203-K

Tang H, Ren Z, Reustle G, Krczal G, Reustle È, Krczal G (2002) Plant regeneration from leaves of sweet and sour cherry cultivars. Sci Hortic 93:235–244. doi:10.1016/S0304-4238(01)00328-4

Thomas P, Swarna GK, Roy PK, Patil P (2008) Identification of culturable and originally non-culturable endophytic bacteria isolated from shoot tip cultures of banana cv. Grand Naine. Plant Cell Tissue Organ Cult 93:55–63. doi:10.1007/s11240-008-9341-9

Trivedi P, Duan Y, Wang N (2010) Huanglongbing, a systemic disease, restructures the bacterial community associated with citrus roots. Appl Environ Microbiol 76:3427–3436. doi:10.1128/AEM.02901-09

Tsavkelova EA, Cherdyntseva TA, Netrusov AI (2005) Auxin production by bacteria associated with orchid roots. Microbiology 74:46–53. doi:10.1007/s11021-005-0027-6

Ulrich K, Stauber T, Ewald D (2008) *Paenibacillus*—a predominant endophytic bacterium colonising tissue cultures of woody plants. Plant Cell Tissue Organ Cult 93:347–351. doi:10.1007/s11240-008-9367-z

Van den Houwe I, Guns J, Swennen R (1998) Bacterial contamination in *Musa* shoot tip cultures. Acta Hortic 490:485–492

Yang HY, Schmidt H (1992) Untersuchungen zur Adventivsproßregeneration in vitro bei Kirschen II. Adventivsproßbildung an in vitro-Blättern verschiedener *Prunus avium*-Idiotypen. Gartenbauwiss 57:7–10

Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B, De Lajudie P (2006) Diverse bacteria associated with root nodules of spontaneous legumes in Tunisia and first report for nifH-like gene within the genera *Microbacterium* and *Starkeya*. Microb Ecol 51:375–393. doi:10.1007/s00248-006-9025-0

Zaspel I, Ulrich A, Boine B, Stauber T (2008) Occurrence of culturable bacteria living in micropropagated black locust cultures (*Robinia pseudoacacia* L.). Eur J Hortic Sci 73:231–235

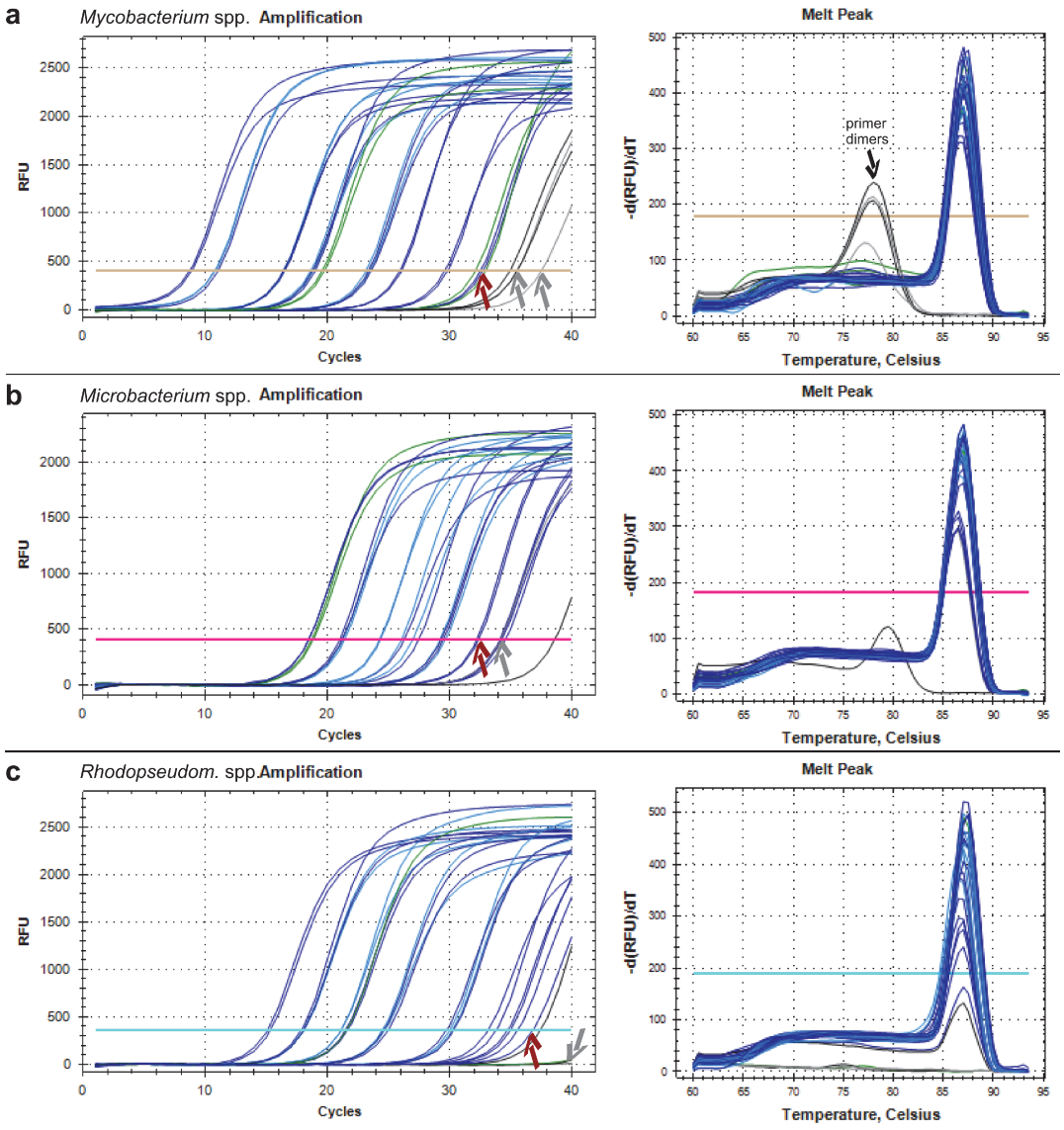
2.3.1 Supplement

Supplement 1 Overview of the origins of the samples used for the quantification of endophytes in *Prunus avium* in vitro cultures.

Genotype	In vitro since	In lab since	Sample harvested	Sampling time
Fama	2004	July 2011	Stem/ leaf during propagation	02/2013
			Stem/leaf/root after rooting	11/2012
			Whole plant, propagation	09/2011
				02/2013
				11/2013
			Adventitious shoots	10/2013 – 02/2014
				02/2014 – 06/2014
Achilleus	2009	August 2011	Stem/ leaf during propagation	02/2013
			Stem/leaf/root after rooting	11/2012
			Whole plant, propagation	09/2011
				02/2013
				11/2013
			Adventitious shoots	10/2013 – 02/2014
				02/2014 – 06/2014
Asteria	1998	August 2011	Stem/ leaf during propagation	02/2013
			Stem/leaf/root after rooting	11/2012
			Whole plant, propagation	09/2011
				02/2013
				11/2013
			Adventitious shoots	10/2013 – 02/2014
				02/2014 – 06/2014
Apollo	1997	August 2011	Stem/ leaf during propagation	02/2013
			Stem/leaf/root after rooting	11/2012
			Whole plant, propagation	09/2011
				02/2013
				11/2013
			Adventitious shoots	10/2013 – 02/2014
				02/2014 – 06/2014
Neptun	2002	July 2011	Stem/ leaf during propagation	02/2013
			Stem/leaf/root after rooting	11/2012
			Whole plant, propagation	09/2011
				02/2013
				11/2013
			Adventitious shoots	10/2013 – 02/2014
				02/2014 – 06/2014
Demeter	1998	August 2011	Stem/ leaf during propagation	02/2013
			Stem/leaf/root after rooting	11/2012
			Whole plant, propagation	09/2011
				02/2013
				11/2013
			Adventitious shoots	10/2013 – 02/2014
				02/2014 – 06/2014

Supplement 2 Regeneration of adventitious shoots from leaf and internode explants on two different culture media.

Explant type	Regeneration medium	Growth regulator	# of explants	Genotype	Regenerated shoots [%]	Shoots per explant [ø]	No of samples for qPCR
Leaf	1	4.5 µM TDZ 1.6 µM NAA	75	Neptun	0	0	0
			75	Demeter	3	1	1
			75	Apollo	4	1.3	3
			75	Asteria	0	0	0
			75	Achilleus	0	0	0
			75	Fama	0	0	0
Internode	1	4,5 µM TDZ 1.6 µM NAA	75	Neptun	0	0	0
			75	Demeter	9	1.7	3
			75	Apollo	9	1	1
			11	Asteria	8	2	0
			75	Achilleus	7	1	0
			75	Fama	1	1	0
Leaf	2	9.1 µM TDZ 2.5 µM IBA	75	Neptun	1	2	2
			75	Asteria	1	1	1
			75	Achilleus	1	1	1
			38	Fama	27	1.3	3



Supplement 3 Determination of the detection limit for the three primer sets to measure **a** *Mycobacterium* spp. **b** *Microbacterium* spp. and **c** *Rhodopseudomonas* spp. in *Prunus avium* in vitro material. Dark blue = Standard curve; light blue = Standard curve with 10 ng cherry DNA added to the reaction; green = plant DNA as positive control (for *Mycobacterium* spp. one with high, one with low bacterial DNA content); grey = control cherry DNA (without the measured bacterium); black = no template control. Red arrow marks the defined detection limit, grey arrow marks the control cherry DNA

3 Discussion

The increased awareness of endophytes has led to the start of numerous research projects during the last years. European networking was additionally stimulated by the COST-Action[5] „Endophytes in Biotechnology and Agriculture“, running from 2011 until 2016. The knowledge about the diversity and function of endophytes has, as a result, increased tremendously and the view of endophytes has changed considerably during the progress of this thesis. This change is especially obvious for tissue culture, where bacteria were first seen as mere contaminations, then accepted as endophytes with neutral effects on plant development. Currently intense research on the diversity and potential growth promoting functions during the propagation process is conducted. At the same time new emerging sequencing technologies gave rise to improved methods for the detection of bacterial communities.

Three different studies, one focusing on the optimization of the propagation protocol, and two on the endophytes in *Prunus avium* in vitro culture, were conducted within this thesis. In this supplementary discussion additional aspects are presented which are not addressed in the respective discussion sections of the manuscripts and help to associate the experiments to the context of current research on endophytes, with the focus on four aspects: (1) alternative methods to detect bacterial communities; (2) the new emphasis on the plant microbiome; (3) a critical view on growth promoting traits in vitro; and (4) potential applications derived from the experimental results. Finally, prospects for future research objectives to study the *Prunus avium*-microorganism interaction are given.

3.1 Alternative methods for the detection of bacterial communities

In this study the bacterial community was assessed by culture-dependent and -independent methods (1.2.5). For the culture-independent detection of bacteria in the plant tissue the ARDRA was chosen as the most suitable method considering the available laboratory equipment and the expected low diversity of bacteria in tissue culture compared to field samples (for details see 3.3). A coverage of over 90% of the estimated total bacterial community was achieved in all analyzed samples with this method (see manuscript II, section 2.2). The ARDRA is based on a 16S rDNA clone library

[5] European cooperation in science and technology (COST)

combined with a restriction digest to sort the obtained plasmids by identical restriction patterns. As the 16S rDNA does not allow the differentiation on species level in all cases, and the method is additionally dependent on the detection of sequence differences by the restriction enzymes, it is possible that the actual bacterial diversity is underestimated. In combination with the isolation of the cultivable fraction of bacteria a validation of the presence or absence of closely related species is possible, however, closely related and non-cultivable species might be overlooked. The major groups detected in the four *Prunus avium* genotypes Fama, Achilleus, Neptun and Demeter by ARDRA were also isolated on bacterial culture media and no additional isolates of the same genera were obtained.

There are several methods that can be used alternatively. Denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) are widely used in microbial ecology including the analysis of endophyte communities, for example by Garbeva et al. (2001); Weinert et al. (2009); Abreu-Tarazi et al. (2010); Videira et al. (2013); Marques et al. (2014). This technique enables the separation of 16S rDNA fragments by increasing denaturing conditions and the detection of nearly 100 % of sequence variants (Muyzer and Smalla 1998). An advantage of the gradient gel methods compared to the clone libraries is the possibility to compare bacterial community profiles of numerous environmental samples (Muyzer and Smalla 1998). Sessitsch et al. (2002) compared DGGE to the clone library approach and confirmed the high resolution of DGGE to up to 1-5 bp nucleotide differences, but still detected additional clones that co-migrated in the gradient gel. Other options are the separation of 16S rDNA in a gel electrophoresis as used in single strand conformation polymorphism (SSCP) and in terminal restriction fragment length polymorphism (T-RFLP) analysis (Overbeek et al. 2006; Smalla et al. 2007; Shen and Fulthorpe 2015).

The named techniques are more and more replaced by next generation sequencing methods (NGS). Compared to clone library analysis, DGGE or T-RFLP, NGS amplicon sequencing allows analysis at greater depth so that more low-abundant taxa can be detected (Knief 2014). A major drawback of NGS is the lower taxonomic resolution compared to Sanger sequencing. While the full length 16S rDNA in most cases enables species identification, NGS reads provide a resolution at maximum down to genus level (Knief 2014). It can be expected that read length will increase due to improvements of the technique in near future (Knief 2014).

All methods for the detection of bacterial communities directly in the host tissue are faced with the problem of a vast amount of host DNA compared to a low amount of bacterial DNA in the samples. During the analysis of bacterial 16S rDNA in plant samples especially the mitochondrial and chloroplast DNA make up a high amount of plasmids in clone benches and amplicons in NGS sequencing due to the endosymbiotic evolutionary origin and the resulting sequence homology. A possibility to reduce the amount of plant organelle DNA in the samples is the use of selective primers instead of universal bacterial primers, e. g. the most commonly used primer 799f published by Chelius and Triplett (2001). This method was successfully applied in this thesis with only 1 of the 380 sequences analyzed in total assigned to chloroplast DNA and no mitochondrial fragments (Quambusch et al. 2014). This selectivity was confirmed in several studies (e. g. (Rasche et al. 2006b; Sun et al. 2008)), but for some host species a low selectivity (Rasche et al. 2006a) or low amplification of bacterial DNA (Shen and Fulthorpe 2015) was reported, and additionally a selective amplification of some bacterial groups cannot be fully excluded (Chelius and Triplett 2001). Shen and Fulthorpe (2015) tested an enzymatic digestion of chloroplast DNA in the PCR template and were able to reduce, but not eliminate, the chloroplast DNA with nearly unchanged bacterial species richness. Another recently published alternative is the use of blocking primers that bind to organelle DNA and prevent their amplification, which resulted in 96 % of bacterial fragments compared to 0.3 % in unblocked samples for the tested plant material (Arenz et al. 2015).

Whole genome shotgun sequencing enables the analysis of the metagenome, providing insight into the composition and physiological potential of whole genomes of the plant and its microbial community (Knief 2014). According to Feehery et al. (2013) this technique can be used as an alternative strategy for assessing microbial diversity in a host. They described a new possibility to circumvent the problem of large amounts of host genomic DNA in the samples. This method uses the differences in the amount of methylation of CpG dinucleotides in bacterial and vertebrate DNA. A complex of magnetic beads, Fc-subunit of human immunoglobulin G and methyl-CpG binding domain specifically interact with the methylated DNA sites and enable the enrichment of bacterial DNA by removal of host DNA bound to a magnet. Feehery et al. (2013) were able to enrich bacterial DNA in human, mouse and black molly fish samples. Problematic for the study of plant metagenomes is again the high amount of chloroplast DNA which has a very low methylation comparable to bacterial DNA (Feehery et al. 2013). In combination with a reduction of organelle DNA, e. g. by blocking primers, this method

would be promising for endophyte research. If the analysis focuses on the RNA transcripts (transcriptome) instead of the genome it provides insight into gene functions and the functional potential of microbial communities.

3.2 The emerging concept of a plant microbiome

Despite of the described limitations NGS technology allows the sequencing of total plant communities in short time and with rapidly decreasing costs. For the first time, it allows the compilation of the collective genomes of microorganisms associated with a plant, and led to the concept of a plant microbiome relating to the well-studied human microbiome (Hardoim et al. 2015). The plant microbiome has been described as an extension of the host phenotype with emerging evidence that the colonization by microbiota can be modulated by the plant (Bednarek et al. 2010). This new perspective gave rise to a number of future research questions concerning plant-microbe interactions. One focus is the analysis of multitrophic interactions, for example the tripartite association of plants, mycorrhiza and bacteria colonizing the fungal hyphae, reviewed by Bonfante and Anca (2009). Another is the documentation and interpretation of shifts in the microbiome, e. g. after inoculation with an endophyte (Ardanov et al. 2012). The question of the influence of the host genotype has, in addition to this thesis (see manuscript II, section 2.2), been addressed in several studies recently. Cardinale et al. (2015) gave a detailed view of the bacterial networks in different lettuce cultivars and found a higher diversity in modern *Lactuca sativa* cultivars than in its wild ancestor *Lactuca serriola*. Differences in the microbiome between potato cultivars were revealed by Marques et al. (2014) and correlated with the starch content.

The idea of a close interaction network of plants and microorganisms is taken one step further in the hologenome theory, in which the holobiont (host plus its microbiota) is seen as a unit of selection in evolution (Rosenberg et al. 2009). Following this theory, genetic variation can occur both in the host and the microbial symbiont genomes, and is therefore not limited to known modes of variation like recombination and mutation, but can also occur by acquiring new traits in form of microbial strains from the environment (Rosenberg et al. 2009).

3.3 Critical view on growth promoting traits during in vitro culture

During in vitro culture, plants are supplied with a sufficient amount and easy accessible nutrients, and the plant hormones in the growth medium are optimized for the specific cultivation step and morphological status of the propagules. This raises the question of

how endophytic bacteria act during in vitro culture. Most of the potential growth promoting traits mentioned in paragraph 1.2.3, especially an increased nutrient availability, nitrogen fixation, and the production of plant hormones, most certainly play a minor role during in vitro culture. Nevertheless, we observed a clear and statistically verified growth promoting effect in terms of a higher percentage of rooted plants and more roots per shoot after the inoculation of the difficult-to-propagate *Prunus avium* genotypes Fama and Achilleus with *Rhodopseudomonas palustris* strain N-I-2 and *Microbacterium testacaeum* strain D-I-1 (see 2.3).

Root organogenesis is highly dependent on the auxin levels in the plant during the different rooting phases (De Klerk et al. 1995) and for both genera *Rhodopseudomonas* and *Microbacterium* strains that produce IAA have been described (Wong et al. 2014; Ji et al. 2014). Kristin Haller tested the auxin production of the strains *Rhodopseudomonas palustris* N-I-2 and *Microbacterium testacaeum* D-I-1 isolated from *Prunus avium* in this study and could show *in vitro* production of a low amount of auxin (27 µg/ml) for the latter only (Quambusch et al. 2016). Studies have shown that IAA synthesis of bacteria is dependent on many factors such as temperature, pH, aerobic/anaerobic conditions and availability of nutrients, and specific biosynthesis pathways can be used according to the environment (Patten and Glick 1996). As a first requisite for plant growth promotion, the auxin production by isolated bacterial strains therefore needs to be confirmed by measurements of endogenous auxin levels to confirm synthesis of the phytohormone *in planta*. Ali et al. (2009) correlated the auxin production by bacterial strains to increased endogenous IAA content of *Triticum aestivum* and Kurepin et al. (2014) measured increased endogenous IAA levels in potato after inoculation with *Burkholderia phytofirmans* along with an increased shoot and root growth. However, during in vitro culture of *Prunus avium*, auxin is supplemented to the growth medium in form of IBA. Label et al. (1989) compared the endogenous IAA content of *Prunus avium* cultures with and without IBA supplementation during the first week of in vitro rooting and observed high levels of IAA in an acropetal gradient in the supplemented variant. In control plants without IBA in the medium, IAA was basipetally distributed and clearly lower than in treated samples. This indicates a strong influence of the IBA content coming from the medium on the endogenous IAA levels and suggests that the influence of IAA produced by bacteria in the plant tissue is, most certainly, negligible. Nevertheless, an effect of endophyte-produced IAA on single cell level during the induction phase of rooting cannot be fully excluded.

Another potential growth promoting factor during in vitro rooting could be a reduction of the ethylene content in the plant tissue by the bacteria. The important role of ACC deaminase in plant growth promotion, and especially in the reduction of high ethylene level produced during plant stress responses, has been highlighted by Glick (2014). De Klerk et al. (1999) reported an inhibitory effect of ethylene during the root induction phase in apple microcuttings with the rooting zone submerged in the medium and gave as a possible explanation that high ethylene levels might accumulate in submerged parts of the stem were aeration is reduced. Additionally, ethylene production is triggered by wounding and by high auxin concentrations (De Klerk et al. 1999) and both conditions apply to the transfer of shoots on rooting medium. Similarly, Biondi et al. (1990) observed a significant inhibition of the rooting percentage of *Prunus avium* microshoots after addition of the ethylene precursor ACC to the medium. During the dedifferentiation phase of rooting the hormone requirements of the plant changes and lower levels of IAA combined with higher ethylene levels are favorable for root formation (De Klerk et al. 1999). The ability to degrade IAA described for some endophytic bacteria (see 1.2.3) could have a growth promoting effect in this phase. Whether ethylene or auxin reduction could play a role in our experimental setup and whether the used bacterial strains are able to reduce these compounds remains to be tested.

In addition to or instead of the direct changes of the plant hormone status, the inoculation with endophytic bacteria could contribute to an equilibrium in the plant microbiome. The addition of a growth promoting or commensal bacterium could act as a counterpart and suppress other bacteria already present in the plant. In our bacterial community study the difficult-to-propagate genotypes Fama and Achilleus were highly dominated by only one bacterial group, the mycobacteria, while easy-to-propagate genotypes Neptun and Demeter each had one additional bacterial group, *Rhodopseudomonas* spp. and *Microbacterium* spp., respectively (see manuscript II, section 2.2). The increased rooting rates after inoculation with the bacterial isolates *Rhodopseudomonas palustris* strain N-I-2 and *Microbacterium testacaeum* strain D-I-1 could be explained by a regulation of the previously dominant mycobacteria to a level which might have been favorable for plant growth (see discussion of manuscript II, 2.2).

The importance of restoring the balance of a bacterial community could play an especially important role during in vitro culture compared to the natural habitat. It can be assumed that the endophyte diversity is strongly reduced by the surface sterilization, the standardized culture conditions and the absence of newly introduced bacteria during

prolonged culture under sterile conditions. This educated guess is supported by many examples with a high diversity detected in field samples e. g. in *Ulmus*, *Coffea*, rice, and *Lactuca* (Mocali et al. 2003; Vega et al. 2005; Sun et al. 2008; Cardinale et al. 2015) compared to a relatively low diversity in tissue culture e. g. in *Robinia*, rockplant, and wild cherry (Zaspel et al. 2008; Koskimäki et al. 2010; Quambusch et al. 2014). Ulrich et al. (2008a, b) analyzed the endophytes of poplar in micropropagated cultures and in field grown trees and observed a highly reduced bacterial community in vitro.

3.4 Outcome of the study and potential applications

3.4.1 *Optimization of micropropagation of* Prunus avium

An optimization of the micropropagation of *Prunus avium* genotype collection silvaSelect© was achieved in close cooperation with the project partner IFP. Rooting was determined as the main limiting factor in the commercial production process. In this thesis the use of a dark treatment to obtain high percentages of rooted plants was established and is already used by the IFP since 2013. The rooting success was improved from 59 % on average over all commercially propagated genotypes in the years 2009-2012 to 79 % in the years 2013-2015 by the proposed method (see manuscript I, section 2.1).

3.4.2 Inoculation with endophytes as a means to improve tissue culture propagation

The potential applications of bacterial endophytes in agriculture and horticulture are plentiful (Compant et al. 2005; Ryan et al. 2008) and several biocontrol agents are available on the market (Berg 2009). Here I want to focus on the outcome of this thesis and possible application of endophytes in micropropagation. A first beneficial outcome of this study is the gained knowledge about the diversity and first insights into the mode of action of endophytes in tissue cultures of *Prunus avium*. In accordance with studies of other plant species the bacterial community was shown to be genotype-dependent (Ardanov et al. 2012; Podolich et al. 2014; Marques et al. 2014; Cardinale et al. 2015) and to have an influence on the growth characteristics of its host plant (Ryan et al. 2008). Beneficial effects of endophytic bacteria during in vitro culture were detected by the inoculation with the two strains *Rhodopseudomonas palustris* strain N-I-2 and *Microbacterium testaceum* strain D-I-1, isolated from easy-to- propagate *Prunus avium* microshoots, as improved rooting of the difficult-to-propagate microshoots (see manuscript II and III, section 2.2 and 2.3).

As one possible application, an inoculum of the isolated bacterial strains could be produced and applied during the transfer on rooting medium in commercial propagation. A simplified inoculation protocol would be a prerequisite for the commercialization. Additionally, the knowledge on the functions of the bacteria within the plant would have to improve to allow the classification into the group of biopesticides or biostimulants according to the legal regulations of the European Union concerning the placing of plant protection products on the market.

Another possibility would be the introduction of the beneficial strains to the plant material only once, during the propagation phase, and market the plant material with an "improved bacterial spectrum". This would circumvent the time-consuming inoculation step. A prerequisite would in this case be the persistence of the bacterial strain within the plant tissue over several subcultures. One obstacle could be the licensing of the technique, to my knowledge there is no exemplary product on the market. A comparable product is the seeds of ryegrass 'Samson AR37' (distributed by Agricom) containing the endophytic fungus *Epichloë* AR37[6] with an optimized alkaloid production to reduce the neurological disease ryegrass staggers of grazing animals and transmit insecticide tolerance.

Possible problems arising from inoculation of in vitro cultures

Bacteria are masters in the adaptation to new and often changing environmental conditions. Many bacteria have a repertoire of several metabolic pathways to enable them to survive both with and without oxygen, light, water, nitrogen and other nutrient sources. For example members of the species *Rhodopseudomonas palustris*, are able to switch between photoautotrophy, photoheterotrophy, chemoautotrophy and chemoheterotrophy to acquire energy and carbon with exceptional flexibility (Larimer et al. 2004). This example demonstrates that the functions endophytic bacteria can have in the plant are not easy to predict and are most certainly diverse and constantly changing depending on abiotic influences, the physiological state of the plant host and competing microorganisms. It also visualizes the possible switch from a plant growth promoting endophytic lifestyle to a saprophytic or even pathogenic lifestyle, and vice versa. The plant is much less flexible concerning the nutrient source, temperature, light conditions and other environmental factors. It therefore remains to be tested, if an inoculation with bacteria leads to a stable culture of *Prunus avium*. As described in 1.2.4, endophytic bacteria can also have a growth inhibiting effect or cover the medium and

[6] Retrieved from: http://www.agricom.co.nz/assets/files/nz/AGC1114SeedGuide2014web.pdf, accessed 24.04.2016

plant tissue after excessive growth, sometimes after prolonged subcultivation or after a change in the culture medium. Additionally, the safety of the sold product, either the bacterial inoculums or the plant material containing bacteria, needs to be assessed, especially with regard to human, animal and plant pathogenicity.

3.5 Future prospects of endophyte research in *Prunus avium* tissue culture

To elucidate the origin of the detected endophytic bacteria a comparison to field grown *Prunus avium* trees would be necessary. The microshoots used in this study had been subcultured for 4 to 16 years at the time of bacterial community analysis. It is not known whether the bacteria have been introduced by the source material and represent part of the naturally occurring population or if they have been introduced by human handling. The original plus trees are not available, but close relatives or *Prunus avium* trees growing at the same site could be analyzed. Winter buds have been used as the source for in vitro culture establishment and therefore should be used in a comparison of the bacterial populations, and NGS of 16S rDNA with the maximum possible read length would be the suggested method.

The localization in the plant tissue can give an indication of the potential mode of action of the bacteria. In this thesis the presence of the bacteria in the different plant organs (leaf, stem, root) has been analyzed (Quambusch et al. 2016). Localization of the bacteria within one tissue could be visualized by in situ hybridization (either with digoxigenin-labeled or fluorophore labeled 16S RNA probes) (Pirttilä et al. 2000; Lo Piccolo et al. 2010), or by GFP tagging of the bacterial strains (Koskimäki et al. 2015). Confocal laser scanning microscopy can be used to visualize cell-cell interactions and can, in combination with co-occurrence studies, be used to unravel microbial interaction networks, as shown for bacteria colonizing lettuce roots (Cardinale et al. 2015). Insights to possible interaction partners within the bacterial community by co-occurrence *in planta* could be used to study the pairwise interactions in detail using co-culture assays.

The effect of inoculation on the bacterial community could be studied by qPCR on selected bacterial strains, or by whole community fingerprinting techniques (DGGE, ARDRA or others). To gain information on the mechanism behind the root inducing effect of the inoculation with *Rhodopseudomonas palustris* and *Microbacterium colombiense* a measurement of the IAA and ethylene content in the plant tissue, especially the root tip, could be conducted. The data could be obtained by high

performance liquid chromatography (Kurepin et al. 2015) or gas chromatography (Ali et al. 2009) combined with mass spectrometry. Mass spectrometry imaging (MSI) provides new tools for the measurement of the spatial distribution of compounds directly on plant tissue sample surfaces (Dong et al. 2016), e. g. by nanospray desorption electrospray ionization mass spectrometry (nanoDESI-MS) (Brader et al. 2014) or laser activated electron tunneling (LAET) used for the measurement of phytohormones in *Cayratia japonica* leaves (Huang et al. 2016). If the isolates are able to degrade ACC and thereby influence the ethylene content in the plant can be tested by ACC deaminase assays using selective media (Jimtha et al. 2014).

In this thesis only bacterial endophytes were analyzed and other microorganisms were excluded from the study. Lucero et al. (2011) analyzed both the bacterial and fungal community of two micropropagated *Atriplex* species and detected fungal cells associated with regenerated leaves and roots by confocal microscopy and diverse fungal taxa in callus by pyrosequencing. Different fungal cells were also detected in embryogenic cultures of *Pinus radiata* (Ganley et al. 2015). Although the fungal endophytes are far less studied than bacteria in tissue culture samples, these two studies are promising examples to suggest an analysis in *Prunus avium*.

4 References

ABREU-TARAZI MF, NAVARRETE AA, ANDREOTE FD, ALMEIDA C V, TSAI SM, ALMEIDA M (2010) Endophytic bacteria in long-term in vitro cultivated "axenic" pineapple microplants revealed by PCR-DGGE. *World J Microbiol Biotechnol* 26:555–560. doi: DOI 10.1007/s11274-009-0191-3

AL BARAZI Z, SCHWABE WW (1985) Studies on possible internal factors involved in determining ease of rooting in cuttings of *Pistacia vera* and *Prunus avium*, cvs Colt and F12/1. *J Hortic Sci* 60:439–445. doi: 10.1080/14620316.1985.11515649

ALI B, SABRI AN, LJUNG K, HASNAIN S (2009) Auxin production by plant associated bacteria: impact on endogenous IAA content and growth of *Triticum aestivum* L. *Lett Appl Microbiol* 48:542–547. doi: 10.1111/j.1472-765X.2009.02565.x

ALI S, CHARLES TC, GLICK BR (2012) Delay of flower senescence by bacterial endophytes expressing 1-aminocyclopropane-1-carboxylate deaminase. *J Appl Microbiol* 113:1139–1144. doi: 10.1111/j.1365-2672.2012.05409.x

AOI Y, KINOSHITA T, HATA T, OHTA H, OBOKATA H, TSUNEDA S (2009) Hollow-fiber membrane chamber as a device for in situ environmental cultivation. *Appl Environ Microbiol* 75:3826–3833. doi: 10.1128/AEM.02542-08

ARDANOV P, SESSITSCH A, HÄGGMAN H, KOZYROVSKA N, PIRTTILÄ AM (2012) Methylobacterium-induced endophyte community changes correspond with protection of plants against pathogen attack. *PLoS One* 7:e46802. doi: 10.1371/journal.pone.0046802

ARENZ BE, SCHLATTER DC, BRADEEN JM, KINKEL LL (2015) Blocking primers reduce co-amplification of plant DNA when studying bacterial endophyte communities. *J Microbiol Methods* 117:1–3. doi: 10.1016/j.mimet.2015.07.003

BEDNAREK P, KWON C, SCHULZE-LEFERT P (2010) Not a peripheral issue: Secretion in plant-microbe interactions. *Curr Opin Plant Biol* 13:378–387. doi: 10.1016/j.pbi.2010.05.002

BELIMOV AA, DODD IC, SAFRONOVA VI, DUMOVA VA, SHAPOSHNIKOV AI, LADATKO AG, DAVIES WJ (2014) Abscisic acid metabolizing rhizobacteria decrease ABA concentrations in planta and alter plant growth. *Plant Physiol Biochem* 74:84–91. doi: http://dx.doi.org/10.1016/j.plaphy.2013.10.032

BERG G (2009) Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. *Appl Microbiol Biotechnol* 84:11–18. doi: 10.1007/s00253-009-2092-7

BIONDI S, DIAZ T, IGLESIAS I, GAMBERINI G, BAGNI N (1990) Polyamines and ethylene in relation to adventitious root formation in *Prunus avium* shoot cultures. *Physiol Plant* 78:474–483. doi: 10.1111/j.1399-3054.1990.tb09066.x

BONFANTE P, ANCA I-A (2009) Plants, mycorrhizal fungi, and bacteria: a network of interactions. *Annu Rev Microbiol* 63:363–83. doi: 10.1146/annurev.micro.091208.073504

BRADER G, COMPANT S, MITTER B, TROGNITZ F, SESSITSCH A (2014) Metabolic potential of endophytic bacteria. *Curr Opin Biotechnol* 27:30–37. doi: 10.1016/j.copbio.2013.09.012

CARDINALE M, GRUBE M, ERLACHER A, QUEHENBERGER J, BERG G (2015) Bacterial networks and co-occurrence relationships in the lettuce root microbiota. *Environ Microbiol* 17:239–252. doi: 10.1111/1462-2920.12686

CARROLL GC (1986) Biology of endophytism in plants with particular reference to woody perennials. In: Fokkema N, Heuvel J van den (eds) Microbiology of the phyllosphere. Cambridge University Press, pp 205–222

CARROLL GC (1988) Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology* 69:2–9.

CHELIUS MK, TRIPLETT EW (2001) The Diversity of Archaea and Bacteria in Association with the Roots of *Zea mays* L. *Microb Ecol* 41:252–263. doi: 10.1007/s002480000087

CLARRIDGE JE (2004) Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev* 17:840–862. doi: 10.1128/CMR.17.4.840

COMPANT S, CLEMENT C, SESSITSCH A (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol Biochem* 42:669–678. doi: 10.1016/j.soilbio.2009.11.024

COMPANT S, DUFFY B, NOWAK J, CLEMENT C, AIT BARKA E (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Appl Environ Microbiol* 71:4951–4959. doi: 10.1128/AEM.71.9.4951

COMPANT S, KAPLAN H, SESSITSCH A, NOWAK J, AIT BARKA E, CLEMENT C (2008) Endophytic colonization of *Vitis vinifera* L. by *Burkholderia phytofirmans* strain PsJN: From the rhizosphere to inflorescence tissues. *FEMS Microbiol Ecol* 63:84–93. doi: 10.1111/j.1574-6941.2007.00410.x

CORNU D, MICHEL M (1987) Bacteria contaminants in shoot cultures of *Prunus avium* L. choice and phytotoxicity of antibiotics. *Acta Hortic* 212:83–86.

COSTACURTA A, VANDERLEYDEN J (1995) Synthesis of phytohormones by plant-associated bacteria. *Crit Rev Microbiol* 21:1–18.

DE BARY A (1866) Morphologie und Physiologie der Pilze, Flechten und Myxomyceten. In: De Bary A, Irmisch T, Pringsheim N, Sachs J (eds) Handbuch der physiologischen Botanik, Vol. II. Wilh. Hofmeister, Leipzig,

DE KLERK GJ, KEPPEL M, TER BRUGGE J, MEEKES H (1995) Timing of the phases in adventitous root formation in apple microcuttings. *J Exp Bot* 46:965–972. doi: 10.1093/jxb/46.8.965

DE KLERK GJ, VAN DER KRIEKEN W, DE JONG J (1999) Review: The formation of adventitious roots: New concepts, new possibilities. *Vitr Cell Dev Biol - Plant* 35:189–199. doi: 10.1007/s11627-999-0076-z

DIAS ACF, COSTA FEC, ANDREOTE FD, LACAVA PT, TEIXEIRA M A., ASSUMPÇÃO LC, ARAÚJO WL, AZEVEDO JL, MELO IS (2008) Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. *World J Microbiol Biotechnol* 25:189–195. doi: 10.1007/s11274-008-9878-0

DÍAZ R, ZAS R, FERNÁNDEZ-LÓPEZ J (2007) Genetic variation of *Prunus avium* in susceptibility to cherry leaf spot (*Blumeriella jaapii*) in spatially heterogeneous infected seed orchards. *Ann For Sci* 64:21–30. doi: 10.1051/forest

DICK JM, LEAKEY RRB (2006) Differentiation of the dynamic variables affecting rooting ability in juvenile and mature cuttings of cherry (*Prunus avium*). *J Hortic Sci Biotechnol* 81:296–302. doi: 10.1080/14620316.2006.11512065

DONG Y, LI B, MALITSKY S, ROGACHEV I, AHARONI A, KAFTAN F, SVATOŠ A, FRANCESCHI P (2016) Sample Preparation for Mass Spectrometry Imaging of Plant Tissues: A Review. Front Plant Sci. doi: 10.3389/fpls.2016.00060

DONNARUMMA F, CAPUANA M, VETTORI C, PETRINI G, GIANNINI R, INDORATO C, MASTROMEI G (2011) Isolation and characterisation of bacterial colonies from seeds and in vitro cultures of *Fraxinus* spp. from Italian sites. *Plant Biol* 13:169–76. doi: 10.1111/j.1438-8677.2010.00334.x

DUCCI F, SANTI F (1997) The distribution of clones in managed and unmanaged populations of wild cherry (*Prunus avium*). *Can J For Res* 27:1998–2004. doi: 10.1139/x97-159

FALEIRO AC, PEREIRA TP, ESPINDULA E, CRISTIANO F, BROD A, BROD FCA, ARISI ACM (2013) Real time PCR detection targeting nifA gene of plant growth promoting bacteria *Azospirillum brasilense* strain FP2 in maize roots. *Symbiosis* 61:125–133. doi: 10.1007/s13199-013-0262-y

FEEHERY GR, YIGIT E, OYOLA SO, LANGHORST BW, SCHMIDT VT, STEWART FJ, DIMALANTA ET, AMARAL-ZETTLER LA, DAVIS T, QUAIL MA, PRADHAN S (2013) A Method for Selectively Enriching Microbial DNA from Contaminating Vertebrate Host DNA. PLoS One. doi: 10.1371/journal.pone.0076096

FERNANDEZ-CRUZ J, FERNANDEZ-LOPEZ J, MIRANDA-FONTAIÑA ME, DIAZ R, TOVAL G (2014) Molecular characterization of Spanish *Prunus avium* plus trees. *For Syst* 23:120–128. doi: 10.5424/fs/2014231-04580

GANLEY RJ, HARGREAVES CL, DONALDSON LA (2015) Detection of asymptomatic fungal microorganisms in *Pinus radiata* tissue culture material. *New Zeal J For Sci* 45:11. doi: 10.1186/s40490-015-0042-y

GARBEVA P, VAN OVERBEEK LS, VAN VUURDE JWL, VAN ELSAS JD, OVERBEEK LS, VUURDE JWL, ELSAS JD (2001) Analysis of endophytic bacterial communities of potato by plating and denaturing gradient gel electrophoresis (DGGE) of 16S rDNA based PCR fragments. *Microb Ecol* 41:369–383. doi: 10.1007/s002480000096

GHANI AKMO, CAHALAN CM (1991) Propagation of *Prunus avium* from root cuttings. *Forestry* 64:403–409. doi: 10.1093/forestry/64.4.403

GLICK BR (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol Res* 169:30–39. doi: 10.1016/j.micres.2013.09.009

GLICK BR, PENROSE DM, LI J (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. *J Theor Biol* 190:63–8. doi: 10.1006/jtbi.1997.0532

HAMMATT N, GRANT NJ (1993) Apparent Rejuvenation of Mature Wild Cherry (*Prunus avium* L.) during Micropropagation. *J Plant Physiol* 141:341–346. doi: http://dx.doi.org/10.1016/S0176-1617(11)81745-1

HAMMATT N, GRANT NJ (1997) Micropropagation of mature British wild cherry. 103–110.

HARDOIM PR, VAN OVERBEEK LS, BERG G, PIRTTILÄ AM, COMPANT S, CAMPISANO A, DÖRING M, SESSITSCH A (2015) The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. *Microbiol Mol Biol Rev* 79:293–320. doi: 10.1128/MMBR.00050-14

HARDOIM PR, VAN OVERBEEK LS, ELSAS JD VAN (2008) Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* 16:463–71. doi: 10.1016/j.tim.2008.07.008

HERMAN EB (1990) Non-axenic plant tissue culture: possibilities and opportunities. *Acta Hortic* 280:233–238.

HILDA R, FRAGA R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol Adv* 17:319–355.

HUANG L, TANG X, ZHANG W, JIANG R, CHEN D, ZHANG J, ZHONG H (2016) Imaging of Endogenous Metabolites of Plant Leaves by Mass Spectrometry Based on Laser Activated Electron Tunneling. *Sci Rep* 6:24164. doi: 10.1038/srep24164

IZUMI H, ANDERSON IC, KILLHAM K, MOORE ERB (2008) Diversity of predominant endophytic bacteria in European deciduous and coniferous trees. *Can J Microbiol* 54:173–179. doi: 10.1139/W07-134

JANßEN A, MEIER-DINKEL A, STEINER W, DEGEN B (2010) Forstgenetische Ressourcen der Vogel-Kirsche/ Forest genetic resources of wild cherry. *Forst und Holz* 65:19–24.

JI SH, GURURANI MA, CHUN S-C (2014) Isolation and characterization of plant growth promoting endophytic diazotrophic bacteria from Korean rice cultivars. *Microbiol Res* 169:83–98. doi: 10.1016/j.micres.2013.06.003

JIMTHA JC, SMITHA P V., ANISHA C, DEEPTHI T, MEEKHA G, RADHAKRISHNAN EK, GAYATRI GP, REMAKANTHAN A (2014) Isolation of endophytic bacteria from embryogenic suspension culture of banana and assessment of their plant growth promoting properties. *Plant Cell Tissue Organ Cult* 118:57–66. doi: 10.1007/s11240-014-0461-0

JOHNSTON-MONJE D, RAIZADA MN (2011) Conservation and diversity of seed associated endophytes in *Zea* across boundaries of evolution, ethnography and ecology. PLoS One. doi: 10.1371/journal.pone.0020396

JONES OP, HOPGOOD ME (1979) The Successful Propagation In Vitro of Two Rootstocks of *Prunus*: the Plum Rootstock Pixy (*P. Insititia*) and the Cherry Rootstock F12/1 (*P. Avium*). *J Hortic Sci* 54:63–66. doi: 10.1080/00221589.1979.11514849

KAYMAK HC (2010) Potential of PGPR in Agricultural Innovations. In: Maheshwari DK (ed) Plant Growth and Health Promoting Bacteria. Springer-Verlag, Heidelberg, pp 45–79

KNIEF C (2014) Analysis of plant microbe interactions in the era of next generation sequencing technologies. *Front Plant Sci* 5:216. doi: 10.3389/fpls.2014.00216

KOBLIHA J (2002) Wild cherry (*Prunus avium* L.) breeding program aimed at the use of this tree in the Czech forestry. *J For Sci* 48:202–218.

KOSKIMÄKI JJ, HANKALA E, SUORSA M, NYLUND S, PIRTTILÄ AM (2010) Mycobacteria are hidden endophytes in the shoots of rock plant [*Pogonatherum paniceum* (Lam.) Hack.] (Poaceae). *Environ Microbiol Rep* 2:619–24. doi: 10.1111/j.1758-2229.2010.00197.x

KOSKIMÄKI JJ, PIRTTILÄ M, IHANTOLA E, HALONEN O, CAROLIN A (2015) extorquens DSM13060 Aggregates around the Host Nucleus and Encodes Eukaryote-Like Proteins. *MBio* 6:1–12. doi: 10.1128/mBio.00039-15

KOZYROVSKA N (2013) Crosstalk between endophytes and a plant host within information processing networks. *Biopolym Cell* 29:234–243.

KUKKURAINEN S, LEINO A, VÄHÄMIKO S, KÄRKKÄINEN HR, AHANEN K, SORVANI S (2004) Occurrence and location of endophytic bacteria in garden and wild strawberry. *HortScience* 39:1–5.

KULKARNI AA, KELKAR SM, WATVE MG, KRISHNAMURTHY K V (2007) Characterization and control of endophytic bacterial contaminants in in vitro cultures of *Piper* spp., *Taxus baccata* subsp. wallichiana, and *Withania somnifera*. *Can J Microbiol* 53:63–74. doi: 10.1139/w06-106

KUREPIN L V., PARK JM, LAZAROVITS G, BERNARDS MA (2015) *Burkholderia phytofirmans*-induced shoot and root growth promotion is associated with endogenous changes in plant growth hormone levels. *Plant Growth Regul* 75:199–207. doi: 10.1007/s10725-014-9944-6

LABEL P, SOTTA B, MIGINIAC E (1989) Endogenous levels of abscisic acid and indole-3-acetic acid during in vitro rooting of Wild Cherry explants produced by micropropagation. *Plant Growth Regul* 8:325–333. doi: 10.1007/BF00024663

LARIMER FW, CHAIN P, HAUSER L, LAMERDIN J, MALFATTI S, DO L, LAND ML, PELLETIER D A, BEATTY JT, LANG AS, TABITA FR, GIBSON JL, HANSON TE, BOBST C, TORRES JLTY, PERES C, HARRISON FH, GIBSON J, HARWOOD CS (2004) Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. *Nat Biotechnol* 22:55–61. doi: 10.1038/nbt923

LEIFERT C, CASSELLS A (2001) Microbial hazards in plant tissue and cell cultures. *Vitr Cell Dev-Pl* 37:133–138. doi: 10.1079/IVP2000129

LEIFERT C, RITCHIE J, WAITES W (1991) Contaminants of plant-tissue and cell cultures. *World J Microbiol Biotechnol* 7:452–469.

LIU T-H A., HSU N-W, WU R-Y (2005) Control of leaf-tip necrosis of micropropagated ornamental statice by elimination of endophytic bacteria. *Vitr Cell Dev Biol - Plant* 41:546–549. doi: 10.1079/IVP2005673

LO PICCOLO S, FERRARO V, ALFONZO A, SETTANNI L, ERCOLINI D, BURRUANO S, MOSCHETTI G (2010) Presence of endophytic bacteria in *Vitis vinifera* leaves as detected by

fluorescence in situ hybridization. *Ann Microbiol* 60:161–167. doi: 10.1007/s13213-010-0023-6

LONG HH, SCHMIDT DD, BALDWIN IT (2008) Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. *PLoS One* 3:e2702. doi: 10.1371/journal.pone.0002702

LUCERO ME, UNC A, COOKE P, DOWD S, SUN S (2011) Endophyte microbiome diversity in micropropagated *Atriplex canescens* and *Atriplex torreyi* var griffithsii. PLoS One. doi: 10.1371/journal.pone.0017693

MANO H, MORISAKI H (2008) Endophytic Bacteria in the Rice Plant. *Microbes Environ* 23:109–117. doi: 10.1264/jsme2.23.109

MARINO G, ALTAN AD, BIAVATI B (1996) The effect of bacterial contamination on the growth and gas evolution of in vitro cultured apricot shoots. *Vitr Cell Dev Biol* 8:51–56.

MARQUES JM, DA SILVA TF, VOLLU RE, BLANK AF, DING G-C, SELDIN L, SMALLA K (2014) Plant age and genotype affect the bacterial community composition in the tuber rhizosphere of field-grown sweet potato plants. *FEMS Microbiol Ecol* 88:424–35. doi: 10.1111/1574-6941.12313

MEIER-DINKEL A (1986) In vitro Vermehrung ausgewählter Genotypen der Vogelkirsche (*Prunus avium* L.). *Allg Forst- u J-Ztg* 157:139–144.

MEIER-DINKEL A, STEINER W, ARTES O, HOSIUS B, LEINEMANN L (2007) Die silvaSELECT-Vogelkirschen- Klonmischung „Escherode I". *AFZ-Der Wald* 5:246–247.

MOCALI S, BERTELLI E, DI CELLO F, MENGONI A, SFALANGA A, VILIANI F, CACIOTTI A, TEGLI S, SURICO G, FANI R (2003) Fluctuation of bacteria isolated from elm tissues during different seasons and from different plant organs. *Res Microbiol* 154:105–114. doi: 10.1016/S0923-2508(03)00031-7

MORTON JB, BENEDITO VA, PANACCIONE DG, JENKS MA (2014) Potential for Industrial Application of Microbes in Symbioses that Influence Plant Productivity and Sustainability in Agricultural, Natural, or Restored Ecosystems. *Ind Biotechnol* 10:347–353. doi: 10.1089/ind.2014.0014

MUYZER G, SMALLA K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* 73:127–141.

NESME X, NORMAND P (2004) Easy individual community typing by rDNA ITS1 analysis. In: Kowalchuk GA, de Bruijn FJ, Head IM, Akkermanns ADL, Van Elsas D (eds) Molecular Microbial Ecology Manual, 2nd ed. Kluwer Academic Publishers, Dordrecht, pp 671–688

OLDROYD GED, MURRAY JD, POOLE PS, DOWNIE JA (2011) The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet* 45:119–44. doi: 10.1146/annurev-genet-110410-132549

OVERBEEK LS VAN, VUURDE J VAN, ELSAS JD VAN (2006) Application of Molecular Fingerprinting Techniques to Explore the Diversity of Bacterial Endophytic Communities. In: Schulz B, Boyle C, Sieber TN (eds) Soil Biology. Springer-Verlag,

Berlin Heildelberg, pp 337–354

PATTEN CL, GLICK BR (1996) Bacterial biosynthesis of indole-3-acetic acid. *Can J Microbiol* 42:207–220. doi: 10.1139/m96-032

PETRINI O (1991) Fungal endophytes of tree leaves. In: Andrews J, Hirano S (eds) Microbial ecology of leaves. Springer, New York, pp 179–197

PIRTTILÄ A, LAUKKANEN H, POSPIECH H, MYLLYLÄ R, HOHTOLA A (2000) Detection of intracellular bacteria in the buds of Scotch pine (*Pinus sylvestris* L.) by in situ hybridization. *Appl Environ Microbiol* 66:3073–3077. doi: 10.1128/AEM.66.7.3073-3077.2000

PIRTTILÄ AM, PODOLICH O, KOSKIMÄKI JJ, HOHTOLA E, HOHTOLA A (2008) Role of origin and endophyte infection in browning of bud-derived tissue cultures of Scots pine (*Pinus sylvestris* L.). *Plant Cell Tissue Organ Cult* 95:47–55. doi: 10.1007/s11240-008-9413-x

PODOLICH O, ARDANOV P, ZAETS I, PIRTTILÄ AM, KOZYROVSKA N (2014) Reviving of the endophytic bacterial community as a putative mechanism of plant resistance. Plant Soil. doi: 10.1007/s11104-014-2235-1

PROMPUTTHA I, LUMYONG S, DHANASEKARAN V, MCKENZIE EHC, HYDE KD, JEEWON R (2007) A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. *Microb Ecol* 53:579–590. doi: 10.1007/s00248-006-9117-x

QUAMBUSCH M, BRÜMMER J, HALLER K, WINKELMANN T, BARTSCH M (2016) Dynamics of endophytic bacteria in plant in vitro culture: quantification of three bacterial strains in *Prunus avium* in different plant organs and in vitro culture phases. Plant Cell Tissue Organ Cult. doi: 10.1007/s11240-016-0999-0

QUAMBUSCH M, PIRTTILÄ AM, TEJESVI M V., WINKELMANN T, BARTSCH M (2014) Endophytic bacteria in plant tissue culture: differences between easy- and difficult-to-propagate *Prunus avium* genotypes. *Tree Physiol* 34:524–33. doi: 10.1093/treephys/tpu027

RASCHE F, TRONDL R, NAGLREITER C, REICHENAUER TG, SESSITSCH A (2006a) Chilling and cultivar type affect the diversity of bacterial endophytes colonizing sweet pepper (*Capsicum anuum* L.). *Can J Microbiol* 52:1036–45. doi: 10.1139/w06-059

RASCHE F, VELVIS H, ZACHOW C, BERG G, VAN ELSAS JD, SESSITSCH A (2006b) Impact of transgenic potatoes expressing anti-bacterial agents on bacterial endophytes is comparable with the effects of plant genotype, soil type and pathogen infection. *J Appl Ecol* 43:555–566. doi: 10.1111/j.1365-2664.2006.01169.x

REINHOLD-HUREK B, HUREK T (2011) Living inside plants: bacterial endophytes. *Curr Opin Plant Biol* 14:435–443. doi: 10.1016/j.pbi.2011.04.004

RIFFAUD J-L, CORNU D, CAPELLI P (1981) Utilisation de la culture in vitro pour la multiplication de merisiers adultes (*Prunus avium* L.) selectionnes en foret (*). *Agronomie* 1:633–640.

ROSENBERG E, SHARON G, ZILBER-ROSENBERG I (2009) The hologenome theory of evolution contains Lamarckian aspects within a Darwinian framework. *Environ Microbiol* 11:2959–2962. doi: 10.1111/j.1462-2920.2009.01995.x

ROSENBLUETH M, MARTÍNEZ-ROMERO E (2006) Bacterial endophytes and their interactions with hosts. *Mol Plant Microbe Interact* 19:827–837. doi: 10.1094/MPMI-19-0827

RUSSELL K (2003) Biology and ecology *Prunus avium*. Technical guideline for genetic conservation and use. Euforgen, Rome, Italy.

RYAN RP, GERMAINE K, FRANKS A, RYAN DJ, DOWLING DN (2008) Bacterial endophytes: recent developments and applications. *FEMS Microbiol Lett* 278:1–9. doi: 10.1111/j.1574-6968.2007.00918.x

SALOMON MV, BOTTINI R, DE SOUZA FILHO GA, COHEN AC, MORENO D, GIL M, PICCOLI P (2013) Bacteria isolated from roots and rhizosphere of *Vitis vinifera* retard water losses, induce abscisic acid accumulation and synthesis of defense-related terpenes in in vitro cultured grapevine. Physiol Plant. doi: 10.1111/ppl.12117

SANTI F, MURANTY H, DUFOUR J, PAQUES LE (1998) Genetic parameters and selection in a multisite wild cherry clonal test. *Silvae Genet* 47:61–67.

SCALTSOYIANNES A, TSOULPHA P, ILIEV I, THERIOU K, TSAKTSIRA M, MITRAS D, KARANIKAS C, MAHMOUT S, CHRISTOPOULOS, V., SCALTSOYIANNES V, ZARAGOTAS D, TZOUVARA A (2009) Vegetative propagation of ornamental genotypes of *Prunus avium* L. *Propag Ornam Plants* 9:198–206.

SCHARDL CL (1996) *Epichloe* species: fungal symbionts of grasses. *Annu Rev Phytopathol* 34:109–30. doi: 10.1146/annurev.phyto.34.1.109

SCHERLING C, ULRICH K, EWALD D, WECKWERTH W (2009) A metabolic signature of the beneficial interaction of the endophyte *Paenibacillus* sp. isolate and in vitro-grown poplar plants revealed by metabolomics. *Mol Plant Microbe Interact* 22:1032–7. doi: 10.1094/MPMI-22-8-1032

SCHLEIFER K-H (2004) Microbial diversity: facts, problems and prospects. *Syst Appl Microbiol* 27:3–9. doi: 10.1078/0723-2020-00245

SCHMID T (2006) *Prunus avium*. In: Roloff A, Weisgerber H, Lang U, Stimm B (eds) Enzyklopädie der Holzgewächse. Wiley, Weinheim, pp 1–16

SESSITSCH A, REITER B, PFEIFER U, WILHELM E (2002) Cultivation-independent population analysis of bacterial endophytes in three potato varieties based on eubacterial and Actinomycetes-specific PCR of 16S rRNA genes. *FEMS Microbiol Ecol* 39:23–32. doi: 10.1111/j.1574-6941.2002.tb00903.x

SHEN SY, FULTHORPE R (2015) Seasonal variation of bacterial endophytes in urban trees. 6:1–13. doi: 10.3389/fmicb.2015.00427

SMALLA K, OROS-SICHLER M, MILLING A, HEUER H, BAUMGARTE S, BECKER R, NEUBER G, KROPF S, ULRICH A, TEBBE CC (2007) Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: Do the different methods provide similar results? *J Microbiol Methods* 69:470–479. doi: http://dx.doi.org/10.1016/j.mimet.2007.02.014

SPAEPEN S, VANDERLEYDEN J, REMANS R (2007) Indole-3-acetic acid in microbial and microorganism-plant signaling. *FEMS Microbiol Rev* 31:425–448. doi: 10.1111/j.1574-6976.2007.00072.x

SUN L, QIU F, ZHANG X, DAI X, DONG X, SONG W (2008) Endophytic bacterial diversity in rice (*Oryza sativa* L.) roots estimated by 16S rDNA sequence analysis. *Microb Ecol* 55:415–424. doi: 10.1007/s00248-007-9287-1

THOMAS P, SWARNA GK, ROY PK, PATIL P (2008) Identification of culturable and originally non-culturable endophytic bacteria isolated from shoot tip cultures of banana cv. Grand Naine. *Plant Cell Tissue Organ Cult* 93:55–63. doi: 10.1007/s11240-008-9341-9

TSAVKELOVA EA, CHERDYNTSEVA TA, NETRUSOV AI (2005) Auxin production by bacteria associated with orchid roots. *Microbiology* 74:46–53. doi: 10.1007/s11021-005-0027-6

TUROK J, COLLIN E, DEMESURE B, ERIKSSON G, KLEINSCHMIT J, RUSANEN M, STEPHAN R (1997) Noble Hardwoods Network. Report of the second meeting. Lourizan, Spain

ULRICH K, STAUBER T, EWALD D (2008a) *Paenibacillus*—a predominant endophytic bacterium colonising tissue cultures of woody plants. *Plant Cell Tissue Organ Cult* 93:347–351. doi: 10.1007/s11240-008-9367-z

ULRICH K, ULRICH A, EWALD D (2008b) Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS Microbiol Ecol* 63:169–80. doi: 10.1111/j.1574-6941.2007.00419.x

VARTOUKIAN SR, PALMER RM, WADE WG (2010) Strategies for culture of "unculturable" bacteria. *FEMS Microbiol Lett* no–no. doi: 10.1111/j.1574-6968.2010.02000.x

VEGA F, PAVA-RIPOLL M, POSADA F, BUYER JS (2005) Endophytic bacteria in *Coffea arabica* L. *J Basic Microbiol* 45:371–380. doi: 10.1002/jobm.200410

VIDEIRA SS, PEREIRA E SILVA MDC, SOUZA GALISA P, DIAS ACF, NISSINEN R, DIVAN VLB, ELSAS JD, BALDANI JI, SALLES JF (2013) Culture-independent molecular approaches reveal a mostly unknown high diversity of active nitrogen-fixing bacteria associated with *Pennisetum purpureum*—a bioenergy crop. Plant Soil. doi: 10.1007/s11104-013-1828-4

WANG B, QIU YL (2006) Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* 16:299–363. doi: 10.1007/s00572-005-0033-6

WANG XM, YANG B, REN CG, WANG HW, WANG JY, DAI CC (2015) Involvement of abscisic acid and salicylic acid in signal cascade regulating bacterial endophyte-induced volatile oil biosynthesis in plantlets of *Atractylodes lancea*. *Physiol Plant* 153:30–42. doi: 10.1111/ppl.12236

WEINERT N, MEINCKE R, GOTTWALD C, HEUER H, GOMES NCM, SCHLOTER M, BERG G, SMALLA K (2009) Rhizosphere communities of genetically modified zeaxanthin-accumulating potato plants and their parent cultivar differ less than those of different potato cultivars. *Appl Environ Microbiol* 75:3859–3865. doi: 10.1128/AEM.00414-09

WONG W-T, TSENG C-H, HSU S-H, LUR H-S, MO C-W, HUANG C-N, HSU S-C, LEE K-T, LIU C-T (2014) Promoting effects of a single *Rhodopseudomonas palustris* inoculant on plant growth by *Brassica rapa* chinensis under low fertilizer input S1. *Microbes Environ* 29:3–4. doi: 10.1264/jsme2.ME14056

WOO PCY, LAU SKP, TENG JLL, TSE H, YUEN KY (2008) Then and now: Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. *Clin Microbiol Infect* 14:908–934. doi: 10.1111/j.1469-0691.2008.02070.x

XIAO Z, JI N, ZHANG X, ZHANG Y, WANG Y, WU T, XU X, HAN Z (2014) The lose of juvenility elicits adventitious rooting recalcitrance in apple rootstocks. Plant Cell, Tissue Organ Cult. doi: 10.1007/s11240-014-0513-5

YANNI YG, RIZK RY, EL-FATTAH FKA, SQUARTINI A, CORICH V, GIACOMINI A, DE BRUIJN F, RADEMAKER J, MAYA-FLORES J, OSTROM P, VEGA-HERNANDEZ M, HOLLINGSWORTH RI, MARTINEZ-MOLINA E, MATEOS P, VELAZQUEZ E, WOPEREIS J, TRIPLETT E, UMALI-GARCIA M, ANARNA JA, ROLFE BG, LADHA JK, HILL J, MUJOO R, NG PK, DAZZO FB (2001) The beneficial plant growth-promoting association of *Rhizobium leguminosarum* bv. *trifolii* with rice roots. *Funct Plant Biol* 28:845–870.

ZAMIOUDIS C, PIETERSE CMJ (2012) Modulation of host immunity by beneficial microbes. *Mol Plant-Microbe Interact* 25:139–150. doi: 10.1094/MPMI-06-11-0179

ZASPEL I, ULRICH A, BOINE B, STAUBER T (2008) Occurrence of culturable bacteria living in micropropagated black locust cultures (*Robinia pseudoacacia* L.). *Eur J Hortic Sci* 73:231–235.

ZHANG D, BERRY JP, ZHU D, WANG Y, CHEN Y, JIANG B, HUANG S, LANGFORD H, LI G, DAVISON P A, XU J, ARIES E, HUANG WE (2015) Magnetic nanoparticle-mediated isolation of functional bacteria in a complex microbial community. *ISME J* 9:603–14. doi: 10.1038/ismej.2014.161

Curriculum vitae

Name:	Mona Quambusch
Date of birth:	20.08.1983
Place of birth:	Flensburg, Germany
Citizenship:	German

Academic education

Since July 2011:	PhD-student in the group of Prof. Dr. Traud Winkelmann, Institute of Horticultural Production Systems, Woody Plant and Propagation Physiology Section, at the Leibniz Universität Hannover
02/2010 – 12/2010	Diploma thesis: Department of Molecular Phytopathology and Mycotoxin Research, Georg-August-Universität Göttingen
	Title: „Untersuchung potentieller Pathogenitätsfaktoren von *Verticillium longisporum* bei der Infektion von *Brassica napus*“
10/2005 – 12/2010	Student of the Georg-August-Universität, Göttingen
	Field of study: Biology
	Specializations: major in botany, minor in phytopathology and biochemistry

School education

08/1994 – 05/2004	Grammar School, Kurt-Tucholsky-Schule, Flensburg-Adelby, Schleswig-Holstein
	Degree: A-levels/ Abitur
08/1990 – 07/1994	Primary School, Husby, Schleswig-Holstein

Awards and Grants

Winner of the *Grow Award 2013* (5000€) granted by the TASPO foundation and Landgard foundation for trendsetting projects in horticultural science. November 2013, Berlin, Germany

Short term scientific mission (STSM) at Oulu University, Department of Biology, in the group of Anna Maria Pirttilä granted by COST Action FA1103 "Endophytes in Agriculture and Biotechnology". November/ December 2012, Oulu, Finland

Best Poster Award granted by the Belgian Plant Biotechnology Association on the conference "Living together: Plant Microorganism Biotechnology". November 2011, Louvain-la-Neuve, Belgium

---

Publications

Quambusch M, Pirttilä AM, Tejesvi M V., Winkelmann T, Bartsch M (2014) Endophytic bacteria in plant tissue culture: differences between easy- and difficult-to-propagate *Prunus avium* genotypes. Tree Physiol 34:524–33. doi: 10.1093/treephys/tpu027

Quambusch M, Brümmer J, Haller K, Winkelmann T, Bartsch M (2016) Dynamics of endophytic bacteria in plant in vitro culture: quantification of three bacterial strains in *Prunus avium* in different plant organs and in vitro culture phases. Plant Cell Tissue Organ Cult. doi: 10.1007/s11240-016-0999-0

Quambusch M, Gruß S, Pscherer T, Winkelmann T, Bartsch M (submitted March 2016) Improved in vitro rooting of *Prunus avium* microshoots by a dark treatment and an auxin pulse. Scientia Horticulturae, under review

---

Conference Presentations

Oral Presentations

Quambusch M, Winkelmann T, Bartsch M (2013) Bacteria associated with plant tissue culture: differences between well and poorly growing *Prunus avium* genotypes. International Symposium "Plant Protection and Plant Health in Europe", Berlin, Germany.

Quambusch M, Winkelmann T, Bartsch M (2013) Endophytic bacteria and plant tissue culture: Differences between easy and difficult-to-propagate *Prunus avium* genotypes. 26. Mitgliederversammlung der Arbeitsgemeinschaft Deutscher In-Vitro-Kulturen (ADIVK), Lünen, Germany.

Quambusch M, Winkelmann T, Bartsch M (2014) Positiver Einfluss endophytischer Bakterien auf die In-vitro-Vermehrung von *Prunus avium*. 49. Gartenbauwissenschaftliche Tagung, Dresden, Germany.

Quambusch M, Haller K, Winkelmann T, Bartsch M (2014) Endophytic bacterial isolates from in vitro shoots enhance in vitro rooting in *Prunus avium*. COST FA1103 work group meeting "Exo- and endogenous signaling", Krakow, Poland.

Poster Presentations

Quambusch M, Winkelmann T, Bartsch M (2011) Characterisation, control and utilisation of bacterial endophytes in in-vitro cultures of *Prunus avium* L. 5. Symposium of the Belgian Plant Biotech Association (BPBA), Louvain-la-Neuve, Belgium.

Quambusch M, Winkelmann T, Bartsch M (2012) Identification of Bacterial Endophytes in In Vitro Cultures of *Prunus avium* using 16S rDNA Sequence Analysis. Workshop of COST Action FA1103 "Endophytes in biotechnology and agriculture", Reims, France.

Quambusch M, Döring M, Winkelmann T, Bartsch M (2012) Bacterial communities associated with *Prunus avium* clones differing in their growth characteristics. Symposium of COST Aktion FA1103 "Endophytes in biotechnology and agriculture", Trento, Italy.

Quambusch M, Pscherer T, Winkelmann T, Bartsch M (2013) Optimierung der In-vitro-Vermehrung und Akklimatisierung von *Prunus avium*: Wie beeinflussen endophytische Bakterien den Produktionserfolg? 48. Gartenbauwissenschaftliche Tagung, Bonn, Germany.

Haller K, *Quambusch M*, Winkelmann T, Bartsch M (2014) Kultivierung und phylogenetische Einordnung endophytischer Bakterienisolate aus *Prunus avium* In-vitro-Kulturen. 49. Gartenbauwissenschaftliche Tagung, Dresden, Germany.

Quambusch M, Winkelmann T, Bartsch M (2015) Use of endophytic bacteria to enhance in vitro rooting in *Prunus avium* shoot cultures. International Symposium "Microbe-Assisted Crop Production-Opportunities, challenges and needs (miCROPe)", Vienna, Austria.

Danksagung/ Acknowledgements

An dieser Stelle möchte ich allen herzlich danken, die mich in der Phase der Promotion begleitet haben:

Liebe Traud, Liebe Melanie, euch möchte ich ganz herzlich für die Überlassung des spannenden Forschungsthemas, die hervorragende Betreuung, vielseitige Anregungen und Unterstützung während der Laborzeit und bei der Entstehung dieser Dissertation danken. Es war einfach toll mit euch zusammen zu arbeiten!

Prof. Dr. Thomas Debener und Prof. Dr. Edgar Maiß, vielen Dank dass sie sich als Korreferent bzw. Prüfungsvorsitz für meine Dissertation zur Verfügung gestellt haben.

Carolin Schneider, Matthias Döring und Silke Gruß vom Institut für Pflanzenkultur möchte ich für die motivierende Zusammenarbeit und interessante Einblicke in die praktische Arbeit eines Unternehmens und außerdem für das zur Verfügung stellen des Pflanzenmaterials danken.

Allen Mitarbeitern der Abteilung Gehölz- und Vermehrungsphysiologie möchte ich ganz herzlich für die schöne Zeit danken. Ohne die fröhliche Zusammenarbeit und den regen Austausch hätte die Arbeit hier mit Sicherheit nur halb so viel Spaß gemacht.

Kristin, Jane, und Toni, euch möchte ich für die motivierte Mitarbeit an unserem Forschungsprojekt im Rahmen eurer Abschlussarbeiten danken. So hat es mir viel Spaß gemacht euch die Arbeit im Labor und die Datenanlyse zu zeigen.

Vielen Dank an Ewa, Frieda, Bärbel, Herrn Geyer und Herrn Barth, die immer dafür gesorgt haben, dass es meinen Versuchspflanzen gut geht und mich bei meinen Versuchen unterstützt haben.

Many thanks to Anna Maria Pirttilä and her group for being my host for the short term scientific mission and giving me the chance to work in a well organized lab with long experience in endophyte research, and for the warm welcome in the very cold Finnish November.

Ich möchte meiner Familie danken, die immer hinter mir steht und mich bei allem unterstützt. Insbesondere danke ich Hannahs Großeltern für die Zeit die ihr in den letzten Monaten auf Spielplätzen und mit Lego verbracht habt, jede Stunde hat mich der Fertigstellung dieser Arbeit ein kleines Stückchen näher gebracht.

Lieber Philipp und liebe Hannah, danke, dass es euch gibt!