Antibiotic treatment impaired growth of tobacco in vitro shoots is associated with oxidative injury and reduced microbiome diversity

Inga Tamošiūnė

Lithuanian Research Centre for Agriculture and Forestry

Elena Andriūnaitė

Lithuanian Research Centre for Agriculture and Forestry

Jurgita Vinskienė

Lithuanian Research Centre for Agriculture and Forestry

Vidmantas Stanys

Lithuanian Research Centre for Agriculture and Forestry

Rytis Rugienius

Lithuanian Research Centre for Agriculture and Forestry

Danas Baniulis (✉ danas.baniulis@lammc.lt)

Lithuanian Research Centre for Agriculture and Forestry

Research Article

Keywords: metagenomic analysis, Mycobacterium, Nicotiana tabacum, timentin

Posted Date: December 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1071558/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Plant in vitro cultures initiated from surface-sterilized explants often harbor complex microbial community. Antibiotics are commonly used to decontaminate plant tissue culture or during genetic transformation, however, the effect of antibiotic treatment on the diversity of indigenous microbial population and consequences for performance of the tissue culture are not completely understood. Therefore, the aim of this study was to assess the effect of antibiotic treatment on the growth and stress level of tobacco (*Nicotiana tabacum* L.) in vitro shoots as well as the composition of plant-associated microbiome. The study revealed that shoot cultivation on medium supplemented with 250 mg L⁻¹ timentin resulted in 29 ± 4% reduced biomass accumulation and 1.2–1.6 fold higher level of oxidative stress injury compared to control. Moreover, the growth properties of shoots were only partially restored after transfer to medium without antibiotic. Shoot microbiome analysis using multi-variable region-based 16S rRNA sequencing revealed abundant microbial community in the control tobacco shoots, including bacteria from 59 families, however, it was largely dominated by *Mycobacteriaceae*. The antibiotic treatment resulted in a decline of species richness (the number of families was reduced 4.5-fold) and increased domination by the *Mycobacteriaceae* family. The results imply that the diversity of plant-associated microbiome might represent a significant factor contributing to the efficient propagation of in vitro tissue culture.

Introduction

Endophytes are a class of endosymbiotic microorganisms that inhabit the internal plant tissues¹. Being protected inside explant or seed tissues, endophytic bacteria evade surface sterilization procedures used for cell culture initiation, and they are common in plant tissues grown in vitro^{2–4}. Some bacterial endophytes maintain habitual concealed lifestyle or due to limited bacterial growth-supporting media conditions remain latent over extended periods of in vitro tissue cultivation. In fact, several studies showed a beneficial effect of endophytic bacteria on the growth of in vitro cultures of tomato⁵, grapevine⁶, sweet cherry⁷, apple⁸, purple coneflower⁹ or tobacco¹⁰. However, the formation of bacterial colonies on culture medium and bacterial overgrowth of plant tissues are rather common and manifest as infection by a variety of pathogenic species or the non-fastidious proliferation of commensal endophytic bacteria that can be triggered by the changes in environmental conditions or plant host physiology^{11–13}.

To eliminate contamination of endophytic origin, pretreatment with high dose¹⁴ or medium supplementation with microbial growth-inhibiting¹⁵ antibiotics have been used and is often reported to improve the propagation or regenerative properties of the tissues^{4, 16–18}. Another common application of antibiotics for in vitro tissue culture is related to the use of *Agrobacterium tumefaciens* bacterium-to-plant DNA transfer machinery as an instrument in the plant genetic transformation¹⁹. Although alternative methods for the selection of genetically modified plants have been developed²⁰, antibiotics remain indispensable for control of *Agrobacterium* growth upon plant tissue culture transformation. Left untreated, *Agrobacterium*, the causal agent of crown gall disease, shows excessive cell proliferation, that

elicits a defense response in plant cells leading to a detrimental effect on plant tissue growth and efficiency of transformation^{21–23}. Cephalosporin and penicillin-type antibiotics, such as cefotaxime, carbenicillin or timentin, active against gram-negative bacteria are commonly used^{24–26}. In culture medium, carbenicillin and penicillins are broken down to physiologically active levels of the auxin-related compounds promoting organogenesis of explants²⁷, however, at higher antibiotic concentration, proline accumulation, oxidative injury and reduced antioxidative activity have been reported indicating elevated stress level²⁸. Previously, low cytotoxic effect of antibiotic timentin on *in vitro* plant tissues has been described^{4,24}, and it is commonly used for transformation procedures in the range of concentrations from 150 mg L⁻¹²⁴ to 500 mg L⁻¹²⁹, sometimes in combination with lower concentrations of other antibiotics³⁰.

Cultivated tobacco (*Nicotiana tabacum* L.) has well established *in vitro* culture conditions³¹ and it has been commonly used as model species for genetic transformation studies as well as for practical application in molecular farming using *in vitro* techniques or contained greenhouse practice^{32–34}. Recently, the composition of the endophytic microbiome of field-grown tobacco plants or seeds has been explored using cultivation and next-generation sequencing-based methods^{35,36} and several endophytic species of the *Bacillus* genus were isolated from tobacco leaves^{10,37}. However, the microbial diversity of *in vitro* cultivated tobacco tissues had not been addressed so far.

Whereas antibiotics find application for explant and tissue culture decontamination or control of bacterial overgrowth during plant transformation, the effect of antibiotic treatment on the composition of endogenous microbial population in tissue culture and the long-term consequences on plant response to stress and growth parameters have not been completely understood. Considering the established role of endophytic bacteria, as modulator of plant growth and adaptation, it could be expected that the antibiotic-induced perturbation of the endophytic community might affect the efficiency of the *in vitro* culture propagation. Therefore, the aim of this study was to assess the effect of antibiotic timentin on growth, stress level of tobacco shoot *in vitro* culture and composition of plant-associated microbiome. Changes of shoot biomass accumulation and oxidative stress injury of cellular membranes during the antibiotic treatment and residual effect following the treatment were assessed. The composition of shoot bacterial population before and after the antibiotic treatment was investigated using a multi-variable 16S rRNA region-based metagenomic analysis. To further refine taxonomic assignment of the dominant taxa that was revealed by the metagenomic analysis, isolate of *Mycobacterium* sp. was obtained using cultivation technique and its antibiotic resistance was tested.

Results

Effect of antibiotic on tobacco shoot growth and oxidative stress injury. Antibiotics could be cytotoxic to plant tissues at concentrations required for efficient control of the bacterium, usually in the range of 100 to 500 mg l⁻¹³⁸. Although low cytotoxic effect of antibiotic timentin on *in vitro* plant tissues had been documented^{4,24,29} and it had been commonly used for plant cell culture at concentrations up to 500 mg

L⁻¹ 24, 29, our experiments showed suppressed growth of tobacco in vitro shoots on medium supplemented with 250 mg L⁻¹ timentin. After 3 weeks of cultivation, an average fresh weight (FW) of the control tobacco shoots (TC) was estimated at 164.7 ± 3.3 mg, and FW of the shoots cultivated on medium containing timentin (TA) was reduced by 29 ± 4% ($p = 1.5 \cdot 10^{-7}$) compared to TC.

An analysis of the membrane lipid oxidative injury of the TC shoots revealed a significant variation of the MDA concentration during the three weeks of the propagation cycle (Fig. 1). The highest value was detected at day 4 after transfer to fresh medium and was estimated at 95.6 ± 2.6 nmol·g⁻¹ FW. The increased MDA accumulation in the control shoots was likely a consequence of the combined effect of stress associated with tissue senescence and shoot injury during the transfer to the fresh medium which was followed by adaptation and active growth on the fresh medium resulting in ~1.3 fold ($p = 4 \cdot 10^{-6}$) decrease in MDA concentration at the end of the first week of cultivation (day 7), and subsequent gradual increase in MDA concentration during the course of culture senescence over the remaining two weeks of the propagation cycle. However, the TA shoots maintained on medium with antibiotic did not follow this cycle and showed 1.2–1.6 fold ($p < 0.014$) higher levels of MDA compared to the TC over the entire period of propagation cycle (Fig. 1) that could be a direct consequence of antibiotic-induced cytotoxic effect.

Interestingly, after transfer to the medium without antibiotic (PA), a consistent residual negative effect of antibiotic treatment on shoot growth vigor and stress level was observed for at least several passages used in the experiments. Cumulative data from several of these experiments revealed that growth vigor of the shoots was only partially restored and accumulation of biomass remained significantly lower (9 ± 3%, $p = 0.013$) compared to TC (Supporting Material Fig. S1). Similarly, the level of MDA accumulation was reduced as compared to TA and showed a significant decrease during the first week after transfer to fresh medium, nevertheless, it remained 1.1–1.3 ($p < 0.033$) fold higher compared to the control over the remaining two weeks of the propagation cycle.

Analysis of bacterial diversity in tobacco in vitro shoot samples. To investigate bacterial diversity in tobacco shoot culture and to assess the effect of antibiotic treatment on the bacterial diversity, DNA extracted from shoots of the TC and PA experimental groups was subjected to metagenomic analysis using the Ion Torrent sequencing platform. Six different DNA extraction and enrichment methods were used for DNA preparation to assess capability to improve the efficiency of the bacterial 16S rRNA metagenomic analysis using as a template the DNA samples derived from the plastid and mitochondrial DNA rich plant material. The PCR amplicon libraries were generated using a multi-variable region approach comprising six regions of the 16S rRNA represented by a combination of five primer pairs (V2, V3, V4, V6-7 and V8) included in the 16S Metagenomic kit (Thermo-Fisher Scientific) (Supporting Material Fig. S2).

The overall number of high-quality mapped sequences with 224-225 bp read length was similar for the TC and PA libraries (1,027,576 and 1,175,938, respectively), but varied up to ~3.5-fold for different DNA preparation methods and primer pairs specific to distinct 16S rRNA gene regions (Supporting Material Table S1 and Fig. S3). The proportion of bacterial sequences varied from 10 to 29% for the different DNA

extraction methods (Supporting Material Fig. S3A). Independent of the experimental group, consistently higher content of bacterial OTUs (>25% of all reads) was detected for samples prepared with DNA extraction methods described by Doyle³⁹ and Ding et al.⁴⁰. For the TC shoots, similar results were also obtained using DNA extraction method described by Li et al.⁴¹. The remaining two methods resulted in a consistently lower proportion of the bacterial OTUs. Bacterial DNA enrichment by selective organelle lysis using SDS and NaCl as previously described by Wang et al.⁴² for preparation of microbial metagenomic libraries of tropic tree *Mallotus nudiflorus* did not increase bacterial OTU content for the tobacco shoot samples as compared to other DNA extraction methods and the approach would require further optimization.

The proportion of sequences assigned to the bacterial and tobacco plastid or mitochondrial 16S rRNA also varied depending on PCR amplification region and/or specificity. Two primer pairs specific to the V4 and V8 regions generated the largest number of reads mapped as bacterial OTUs and the largest proportion of the bacterial OTUs as compared to the overall number of reads (Supporting Material Fig. S3B). Meanwhile, the overall amplification efficiency of the V6-7 and V2 primers was 2- to 4-fold lower, the resulting proportion of bacterial sequences was comparable to the V8. The V3 primers generated a large number of reads which was comparable to V8 but resulted in the lowest proportion of bacterial sequences. Previously, the V3 region had been shown to efficiently represent the diversity of microbial communities of the fecal or sewage samples^{43–45}, however, our analysis using plant-derived samples showed and V3 primers included in the 16S Metagenomics Kit showed a strong predisposition toward plastid and/or mitochondrial sequences. It is notable that, for all primer sets, a consistently lower ratio of bacterial to plastid/mitochondrial OTUs was obtained for the PA as compared to the TC sample which could be a result of lower yields of bacterial DNA due to lower bacterial density in the antibiotic-treated shoot sample.

Principal coordinate analysis (PCoA) of the microbiome data sets using Bray–Curtis dissimilarity matrix represented variation mainly between the two tobacco shoot experimental groups on the first coordinate and variation resulting from the application of multi-variable region 16S analysis were plotted mainly on the second coordinate (Fig. 2). Although larger variation was observed among the primer pairs for the PA sample, the overall pattern was very similar to that observed for the TC sample where V2 and V4 data sets represented the largest difference. The larger variation within the PA group was likely the result of a 5.6-fold lower number of OTUs mapped as compared to the TA sample (Supporting Material Table S1).

The region-specific primer capability to represent bacterial taxonomic diversity was assessed using rarefaction and alpha diversity analysis. A number of reads representing bacterial OTUs of the V3 data set were insufficient for the rarefaction analysis. For the remaining four regions the saturated curves indicated that the bacterial diversity was sufficiently represented and a several-fold difference in species richness between the two experimental groups, TC and TA, was revealed (Supporting Material Fig. S4). Alpha diversity analysis revealed a similar distribution of the Richness measure and the related Chao1 and Fisher's diversity indices (Supporting Material Table S2 and Fig. S5) among the data sets generated using distinct primer pairs. A significant difference was detected for the Evenness measure due to the

high value estimated for the V3 region which appeared mainly related to the under-representation of the *Mycobacteriaceae* family in the V3 data set (0.2 and 40% of bacterial OTUs for the TC and PA, respectively) as compared to other primer pairs where the family was largely dominant (79-90% and 94-98%, respectively) (Supporting Material Table S3; Fig. 4).

To evaluate 16S rRNA region-specific primer propensity for specific taxonomic groups, family-level data was used to avoid bias due to the limited accuracy of genus or species identification using short-read sequences which could lead to underestimation of the primer specificity. The association among the OTUs obtained using different primer pairs was mapped on the UpSet plot using cumulative data from both experimental groups (Fig. 3). Among the 59 family level OTUs, 22% were detected by all primer pairs and were represented by 96% or 70% of all bacterial reads when the dominant *Mycobacteriaceae* family was included or excluded from the analysis, respectively. No singleton families were detected using V2-specific primers and 26 (42% of all families) singleton families were detected by the remaining four primer pairs, however, these included only 0.4% of the total number of reads mapped to bacterial OTUs. *Chitinophagaceae* and *Sinobacteraceae* (880 and 560 reads, respectively) were the most abundant singleton families detected using V4- and V8-specific primers, meanwhile, the remaining singletons included OTUs of relatively low abundance (<100 reads). The results indicate that the multi-variable 16S rRNA region-based metagenomic analysis approach is capable to provide higher taxonomic resolution of microbial diversity as compared to results obtained using individual regions, especially for the low abundance taxa.

Previously, a variation of informative power for the same 16S rRNA region-specific primers was demonstrated in the study with human intestinal or environmental microbiota samples⁴⁵⁻⁴⁷. In our study, the read abundance for the same OTU also varied considerably among the primer pairs (Fig. 4). For example, the most abundant *Mycobacteriaceae* family was represented by 37%, 18% and 39% of the total number of reads using V4, V6-7 and V8 primers, meanwhile, only 6% and <1% of the total number of reads were assigned to the family for the V2 and V3 primer data sets, respectively. In another instance, the distribution of reads assigned to the *Paenibacillaceae* family among V3, V4 and V8 data sets was 15%, 38% and 44%, respectively, meanwhile, V2 and V6-7 represented only 3% and <1% of the total number of reads, respectively. Regardless of the apparent taxa-specific amplification efficiency among the primer pairs, the hierarchical cluster analysis of the data did not reveal consistent distribution of taxonomic groups among the primer data sets (Fig. 4), possibly due to variation of primer specificity at lower taxonomic level or the bias introduced by variation of the taxa abundance. Nevertheless, the results imply that cumulative data obtained from several primer pairs with different taxa-specific amplification efficiency has limited quantitative capacity to represent species abundance variation (species evenness) within a sample, however, this would not affect the quantitative estimate of inter-sample abundance variation for specific OTU.

Antibiotic effect on bacterial diversity in tobacco shoot culture. Although both, the TA and PA, samples had a similar overall number of reads and reads mapped to bacterial OTUs, the number of unique OTUs was reduced from 153 to 27 upon the antibiotic treatment (Supporting Material Table S1). The antibiotic

effect resulted in 42% of total variability of the microbiome data between the TA and PA experimental groups as represented by the first coordinate of the PCoA (Fig. 3), and alpha diversity analysis showed a significant reduction in species richness and evenness upon the antibiotic treatment (Fig. 5).

Among the six phyla detected in the tobacco shoot microbiome, *Actinobacteria* was dominant in both experimental groups and was mainly represented by the order of *Actinomycetales* including 17 and 3 families for the TC and PA experimental groups, respectively (indicated in a blue color font in Fig. 4; Supporting Material Table S3). It is remarkable that *Actinobacteria* included the most prevalent family of *Mycobacteriaceae*, representing 81% and 98% of mapped bacterial reads for the TC and PA, respectively. *Microbacteriaceae* represent another antibiotic treatment enduring actinobacteria that became the second most abundant OTU with a relative abundance of 1.6% in the antibiotic-treated shoot sample, meanwhile, *Propionibacteriaceae* and *Acidimicrobiaceae* were detected only at the marginal level. For the latter OTU, sequence comparison showed 98% sequence similarity to *Mycobacteriaceae* which could also imply inaccuracy of the sequence assignment. A similar assumption could be drawn for the assignment of *Thermolithobacteraceae* family of the phylum *Firmicutes*. Meanwhile, *Bacillaceae* and *Paenibacillaceae* were the most abundant among the remaining four families of *Firmicutes* representing 3% of mapped bacterial reads in the control shoots. In addition, *Staphylococcaceae* and *Streptococcaceae* were detected at low abundance but by three primer pairs each. The abundance of all *Firmicutes* was largely reduced (<0.1% of mapped reads) upon the antibiotic treatment.

In the control shoots, 1.6% of mapped reads were assigned to 5 families of *Bacterioidetes*, mainly represented by *Sphingobacteriaceae*, *Chitinophagaceae* and *Flavobacteriaceae*, but all of them were undetectable in the antibiotic-treated sample. Among the *Proteobacteria* including 8% and 0.5% of mapped bacterial reads of the TC and PA, respectively, class *Alpha-proteobacteria* included 10 families of order Rhizobiales representing many well-known beneficial plant-associated bacteria ⁴⁸, and also *Caulobacteraceae* and *Sphingomonadaceae* families including common environmental bacteria ^{49, 50}. In the antibiotic-treated shoots only *Caulobacteraceae* and *Bradyrhizobiaceae* were detected at 4- and 10-fold reduced abundance level compared to control shoots, respectively. Interestingly, families *Kopriimonadaceae*, *Rhodobacteraceae* and *Rhodospirillaceae* including species of common environmental and aquatic bacteria were detected at low abundance only in the antibiotic-treated shoots.

The *Desulfovibrionaceae* family of class *Deltaproteobacteria* was detected at low but similar abundance in both, TC and PA, shoots. However, more proliferous *Beta-proteobacteria* and *Gamma-proteobacteria* (including 5 and 7 families, respectively) were also largely reduced upon the antibiotic treatment. Among the *Beta-proteobacteria* were notable *Alcaligenaceae* and *Methylophilaceae* which include nitrifying and methylotrophic environmental bacteria ^{51, 52}. Meanwhile families *Enterobacteriaceae* and *Pseudomonadaceae* of *Gamma-proteobacteria* represent numerous plant endophytes or pathogens ⁵³. In addition, two families of the PVC superphylum, *Planctomycetes* and *Verrucomicrobia*, which includes common soil and plant root-associated bacteria ⁵⁴ were both detected at low abundance in the control shoots.

Isolation of Actinobacteria and antibiotic resistance assessment. To further characterize the actinobacteria dominant in the in vitro tobacco shoot culture, the extract of antibiotic-treated shoots was plated on the Loewenstein-Jensen growth medium. The isolate obtained from bright yellow colonies forming after 6-8 weeks of incubation showed 98% identity to the *M. cookii* strain ATCC 49103 (GenBank accession NR_114661.1)⁵⁵ based on the 1407 nt fragment of 16S rRNA. The isolate also sustained similar growth properties on the Actinobacteria Isolation Agar which was used for later cultivation and antibiotic resistance test. The isolate showed resistance to timentin at the concentration used for the tobacco shoot treatment (250 mg·L⁻¹) and chloramphenicol at 30 mg L⁻¹ (Supporting Material Fig. S6), however, the growth of the isolate was suppressed by rifampicin at 25 mg L⁻¹ which could potentially be used for the mycobacterium elimination from the plant in vitro culture.

Discussion

An abundant microbial community of the control tobacco shoots was revealed by metagenomic analysis using a multi-variable 16S rRNA region approach and 153 OTUs representing 59 families of the bacteria domain were detected (Fig. 4). Bacterial endophytes are common in the in vitro cultures initiated from surface-sterilized plant dormant tissues, such as seeds or buds, which contain a complex community of endophytic bacteria. Previously, the study by Thomas et al.⁵⁶ revealed a vast diversity of endophytic bacteria prevailing in grapevine field shoots (mainly *Proteobacteria* but also *Actinobacteria*, *Firmicutes* and several less abundant phyla) and their introduction to in vitro shoot culture was demonstrated. The occurrence of endophytic bacteria in seeds is also well documented^{57, 58}. Since the tobacco in vitro culture used in our study was initiated from surface-sterilized seeds, it is possible that numerous bacterial species survived the sterilization procedure in internal tissues or even on the surface of seeds. Endophytic species vertically transmitted through seed are closely associated with plant host and often play a significant role in the regulation of plant growth and development physiology. Previously, *Enterobacteriaceae* was detected as the predominant species of the endophyte community of tobacco seeds of the four distinct tobacco cultivars, and a genotype-specific signature was observed for *Alpha-proteobacteria*³⁶. In our study, among the bacteria identified in shoot microbiome, numerous species of *Bacillales*, *Rhizobiales*, *Burkholderiales* or *Enterobacteriales* could include plant endophytic bacteria adapted for vertical transmission and capable to inhabit seed tissues.

Alternatively, endospore or biofilm-forming bacteria are adapted to survive in extreme environments, many show resilience to sterilizing agents and would endure seed sterilization procedures. Such route could be adequate for soil bacteria such as *Mycobacterium* spp. which forms resilient endospores⁵⁹, are capable to colonize plant tissues but have not been detected in seeds so far^{60, 61}. Similarly, species of *Sphingomonadaceae* are widely distributed in nature, having been isolated from many different land and water habitats, as well as from plant root systems and could form resilient biofilms which often are capable to survive sterilization conditions⁶².

Despite the multi-variable region metagenomic analysis used in our study, it had limited capacity to reflect intra-sample abundance differences among the taxa. It appeared that the control tobacco shoot microbiome was largely dominated by *Mycobacteriaceae*, and likely one of its dominant species was shown to be closely related to *Mycobacterium cookii*. *Mycobacteria* are widely distributed in water and soil ⁶³, however, despite the fact that the genus *Mycobacterium* comprises nearly 200 species, so far the research has mainly focused on obligate pathogens and information about plant-associated mycobacteria is relatively scarce. *Mycobacterium* spp. have been isolated from the rhizosphere of tomato ⁶⁴ and rice plants ⁶⁵. They have also been detected by sequencing-based analysis as endophytes of rice roots and stems ⁶¹, wheat roots ⁶⁶, shoots of rock plant ⁶⁷ and buds of Scots pine ⁶⁸. Although previously, *Mycobacterium* spp. have not been described as tobacco endophyte, it might not represent an exception and might vary depending on cultivation conditions. In particular, endophytic or epiphytic colonization would be plausible for the plants cultivated on peat substrate considering that mycobacteria are abundant in peat as they are common inhabitants of sphagnum vegetation ⁶⁹, which was also one of the first described habitats for *M. cookii* ⁷⁰.

It is notable that previously *Mycobacterium* spp. have been detected as dominant species in sweet cherry in vitro shoots ⁷¹ and tissue cultures of Scots pine ⁷². Although in the latter case, some species of *Mycobacterium* have been reported to suppress undifferentiated Scots pine tissue growth and reduce the length of hypocotyls of seedlings, no negative effect has been observed for other species ⁷². However, plant growth regulating properties have not been described for species related to *M. cookii* so far.

In our study, antibiotic timentin treatment had a detrimental effect on the endophytic community of tobacco in vitro shoots. The number of detected families was reduced ~4.5-fold and most of the remaining families had largely reduced counts. Interestingly, antibiotic treatment had little effect on the abundance of the dominant *Mycobacteriaceae* and *Microbacteriaceae* families (Supporting Material Table S3) as well as two *Alpha-proteobacteria* families, *Caulobacteraceae* and *Bradyrhizobiaceae*, that showed a modest reduction in relative abundance. Recently, the antibiotic effect on the diversity and structure of bacterial communities was described in the soil environment ⁷³. The study revealed that tetracycline addition could change the microbial community composition and relative abundance of bacteria due to a combination of growth suppressing or bactericidal effect of tetracycline and the induction of bacterial antibiotic resistance. In our study, the antibiotic resistance of the *M. cookii* related isolate supported the notion that increased domination of the *Mycobacteriaceae* family in the timentin treated shoots is a consequence of antibiotic resistance. Resistance to carboxypenicillins, such as ticarcillin, had been well documented for a variety of bacteria including actinobacterial taxa ⁷⁴. In addition, the resistance to clavulanic acid inhibitor would be required to ensure effective growth observed for the *Mycobacterium* isolate, which is relatively common among bacteria as well ⁷⁵. Resistance to antibiotics is also the most likely explanation for the unchanged or moderately decreased abundance of other bacterial taxa detected in the antibiotic-treated shoots, however, this would require further experimental confirmation.

The antibiotic-induced perturbations in composition and/or interactions within the plant-associated microbial community of the tobacco shoots could be a significant factor contributing to the enduring negative effect on the growth and adaptive capacity of the plant tissue culture. Plant microbial community has a profound effect on the health of the host plant and reduced microbial diversity often leads to disease development^{76,77}. It could be proposed that the antibiotic-induced perturbations in microbial community structure could eliminate pathogen suppressing antagonistic interactions and facilitate the spread of pathogenic microorganisms or disease susceptibility of the plants could be increased due to elevated stress level. However, the antibiotic-treated tobacco shoots did not show symptoms that could be attributed to microbial pathogenesis, such as distorted morphology or necrosis of tissues (Supporting information, Figure S1). Alternatively, a loss of mutualistic interaction with beneficial endophytic bacteria could contribute to reduced adaptation to in vitro conditions and inhibited shoot growth. Indeed, growth-promoting^{5,9} stress-reducing activity^{6,8,10} of bacteria on in vitro propagated plants have been reported previously. A large diversity of bacteria affected by the antibiotic treatment detected in our analysis would lead to an extensive list of potential candidates for the beneficial interaction, therefore, further studies, potentially including specific bacterial isolates or microbial consortia, would be required to refine the role of plant microbiome in modulation of in vitro culture growth and adaptation to the in vitro environment.

Conclusion

Our study revealed that the antibiotic timentin induced suppression of growth and elevated stress level of tobacco in vitro shoots is followed by enduring residual negative effect after shoot transfer to medium without antibiotic. The analysis of the control tobacco shoot microbiome detected an extensive bacterial community dominated by the *Mycobacteriaceae* family which potentially could originate from seeds used to initiate the in vitro culture and might include endophytic species closely associated with the plant host as well as contamination of resilient environmental bacteria. The antibiotic treatment-induced decline of species richness, increased domination of *Mycobacteriaceae* and several other families were likely associated with antibiotic resistance traits of the bacteria. It was proposed that antibiotic-induced perturbation of shoot microbiome composition and/or interactions might contribute to the reduced adaptive capacity and impede the growth of the tobacco shoots leading to the reduced efficiency of the in vitro culture propagation. Therefore, further insights into the specific role of the members and interactions of the tobacco shoot microbiome would ensure a better understanding of the antibiotic effect on the in vitro tissue as holobiont and would potentially provide solutions required to improve the efficacy of the in vitro tissue culture.

Methods

Tobacco shoot culture in vitro and antibiotic treatment. Cultivated tobacco (*Nicotiana tabacum* cv. Samsun-NN) in vitro shoot culture was derived from seeds kindly provided by Prof. J.P.T. Valkonen (University of Helsinki). Plants were cultivated and all experiments were carried out in accordance with

relevant guidelines. Tobacco shoots cultivated in vitro for over 5 years were used as a control (TC) and were maintained on solid in Murashige-Skoog (MS) medium⁷⁸, supplemented with 0.75 mg·L⁻¹ 6-benzylaminopurine, 30 g·L⁻¹ sucrose, 0.8% agar at 25 ± 1 °C, under 150 μmol·m⁻²·s⁻¹ intensity illumination of 16 h photoperiod. Antibiotic-treated tobacco shoots (TA) were maintained as described for the TC, except the medium was supplemented with timentin at 250 mg·L⁻¹. To ascertain stable and homogeneous physiological response in the shoot culture, the duration of the treatment was 6 months. Prolonged cultivation was also used to clear residual DNA from antibiotic-inactivated bacteria to avoid false-positive results during the shoot microbiome analysis based on metagenomic DNA sequencing. After transfer to medium without antibiotic (PA), the shoots were maintained under the same conditions as TC and at least one culture passage (one-month duration) was used before collecting samples for the analysis.

Samples for shoot fresh weight (FW) and microbial composition analysis were collected three weeks after transfer to fresh medium, and oxidative stress injury was assessed at four distinct time points during the propagation cycle.

Assessment of oxidative stress injury. Oxidative injury of tobacco shoot cellular membranes was estimated based on quantitative analysis of the lipid peroxidation product malondialdehyde (MDA) using previously described method^{79,80}. Homogenized frozen tobacco shoot powder was extracted with 50mM Tris-HCl pH 7.4, containing 1.5% of polyvinylpolypyrrolidone for 30 min at 4°C and centrifuged at 10,000 ·g for 15 min at 4°C. Equal amounts of tissue extract and 0.5% thiobarbituric acid in 20% trichloroacetic acid were mixed, heated at 95 °C for 30 min, cooled on ice and centrifuged at 10,000 ·g for 5 min. The absorbance measured at 532 nm was corrected by subtracting the absorbance value at 600 nm and MDA concentration was estimated using $\epsilon = 155 \text{ mM}^{-1}\text{cm}^{-1}$. The absence of interference from the absorbance of anthocyanins at 532 nm was verified using control samples without thiobarbituric acid.

Bacterial 16S RNA metagenomic analysis. The metagenomic analysis samples from the TC and PA experimental groups were fresh frozen in liquid N₂ and stored at -70°C. To assess methods most efficient for bacterial DNA extraction from plant material, six different DNA extraction methods described by^{39,40,81,82}, PureLink Microbiome DNA Purification kit (Thermo Fisher Scientific, USA) and a method for bacterial DNA enrichment using SDS detergent extraction⁴² were used. DNA libraries were prepared using five primer pairs from the Ion 16S Metagenomics kit (Thermo Fisher Scientific, USA) (Supporting Material Fig. S2) as described previously⁸³. Equal volumes of all DNA library samples (adjusted to 10 pM) were combined and emulsion PCR was carried out using Ion OneTouch 2 System and Ion PGM Hi-Q View OT2 Kit (Thermo Fisher Scientific, USA). The clonal libraries were enriched using Ion OneTouch ES (Thermo Fisher Scientific, USA) and sequencing was performed using Ion 316 v.2 chip on the Ion Personal Genome Machine system using Ion PGM Hi-Q Sequencing kit (Thermo Fisher Scientific, USA). Base-calling and run demultiplexing were performed by Torrent Suite v.5.0.5 (Thermo Fisher Scientific, USA) with default parameters. Sequencing data were processed using 16S Metagenomic workflow of the Ion Reporter Software v.5.10.5.0 (Thermo Fisher Scientific, USA). Reads were trimmed by primers at both ends. The

threshold for unique reads was set to 10. Taxonomic identification was performed using MicroSEQ 16S Reference Library v.2013.1 and Greengenes v.13.5 databases and *N. tabacum* plastid 16S rRNA (NC_001879.2:102762-104252) and mitochondrion 18S rRNA (NC_006581.1:108334-110235) sequences were included to detect sequences resulting from contamination of the plant mitochondrial and plastid host DNA. The threshold value for percentage identity for genus and species ID was 97% and 99%, respectively.

Mycobacterium isolation, identification and antibiotic resistance test. Tobacco shoots were homogenized with a razor blade in MS medium, the homogenate was applied to Loewenstein-Jensen medium prepared from TB-Medium base (Merck, Germany) and incubated at room temperature for 6-8 weeks. The bacteria isolate was maintained on the Actinobacteria Isolation Agar (AIA). Bacterial DNA was isolated using the GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific, USA). The 16S rRNA gene fragment was amplified using the universal primers as described previously¹⁰ and the sequence was queried at the NCBI BLAST server⁸⁴. Isolate resistance to antibiotics was tested by cultivation on AIA medium supplemented with timentin (250 mg·L⁻¹), chloramphenicol (30 mg·L⁻¹) or rifampicin (25 mg·L⁻¹).

Statistical data analysis. Statistically significant differences of shoot FW and MDA accumulation were assessed by ANOVA analysis and Tukey post hoc test using IBM SPSS Statistics v.21 (IBM Inc.). Data are presented as the mean of at least 3 independent experiments and standard error of the mean.

Statistical analysis of the metagenomic data and visualization was performed using PCoA, Rarefaction, Alpha diversity and HeatMap functions of the Calypso web-based platform⁸⁵. Total sum normalization was applied to taxonomic counts data by dividing feature read counts by the total number of reads in each sample. To account for the non-normal distribution of the data, cumulative-sum scaling and log2 transformation were used⁸⁶. The representation of the microbiome by the primer pairs representing distinct rRNA regions was visualized using UpSet graph⁸⁷.

Declarations

Acknowledgements

This project has received funding from European Regional Development Fund (project No. 01.2.2-LMT-K-718-01-0037) under grant agreement with the Research Council of Lithuania (LMTLT). The authors would like to thank Dr. D. Gelvonauskienė, Dr. P. Haimi for assistance and constructive discussions and Ms. Z. Kajatienė for valuable technical support.

Author Contributions

I.T., E.A., R.R., V.S., D.B. conceptualized and designed the experiments; I.T., E.A., J.V., R.R. acquired, analyzed and interpreted the data; I.T., E.A., D.B. drafted the manuscript; R.R., V.S. contributed to critical revision of the manuscript. All authors reviewed the manuscript.

Competing Interests: The authors declare no competing interests

References

1. Brader, G. *et al.* Ecology and genomic insights into plant-pathogenic and plant-nonpathogenic endophytes. *Annu. Rev. Phytopathol*, **55**, 61–83 (2017).
2. Kukkurainen, S. *et al.* Occurrence and location of endophytic bacteria in garden and wild strawberry. *HortScience*, **40**, 348–352 (2005).
3. Abreu-Tarazi, M. F. *et al.* Endophytic bacteria in long-term *in vitro* cultivated “axenic” pineapple microplants revealed by PCR–DGGE. *World J. Microbiol. Biotechnol*, **26**, 555–560 (2010).
4. Leone, G. F. *et al.* Use of antibiotics to control endophytic bacterial growth migration onto culture medium in *Eucalyptus cloeziana* F. Muell.: a micropropagation approach. *In Vitro Cell. Dev. Biol. Plant*, **55**, 421–432 (2019).
5. Botta, A. L., Santacecilia, A., Ercole, C., Cacchio, P. & Del Gallo, M. *in vitro* and *in vivo* inoculation of four endophytic bacteria on *Lycopersicon esculentum*. *N. Biotechnology*, **30**, 666–674 (2013).
6. Salomon, M. V. *et al.* 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*, **151**, 359–374 (2014).
7. Quambusch, M., Brümmer, J., Haller, K., Winkelmann, T. & Bartsch, M. 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 Tiss. Org. Cult*, **126**, 305–317 (2016).
8. Tamosiune, I. *et al.* Endophytic *Bacillus* and *Pseudomonas* spp. modulate apple shoot growth, cellular redox balance, and protein expression under *in vitro* conditions. *Front. Plant Sci*, **9**, 889 (2018).
9. Maggini, V. *et al.* Tissue specificity and differential effects on *in vitro* plant growth of single bacterial endophytes isolated from the roots, leaves and rhizospheric soil of *Echinacea purpurea*. *BMC Plant Biol*, **19**, 284–284 (2019).
10. Andriūnaitė, E. *et al.* Stimulation of *Nicotiana tabacum* L. *in vitro* shoot growth by endophytic *Bacillus cereus* group bacteria. *Microorganisms*, **9**, 1893 (2021).
11. Cassells, A. C.. Problems in tissue culture: culture contamination, in Micropropagation: Technology and Application. (eds. P.C. Debergh & R.H. Zimmerman)31–44(Springer Netherlands, Dordrecht; 1991)
12. Reed, B. M., Buckley, P. M. & DeWilde, T. N. Detection and eradication of endophytic bacteria from micropropagated mint plants. *In Vitro Cell. Dev. Biol. Plant*, **31**, 53–57 (1995).
13. Cassells, A. C. & Tahmatsidou, V. The influence of local plant growth conditions on non-fastidious bacterial contamination of meristem-tips of *Hydrangea* cultured *in vitro*. *Plant Cell Tiss. Org. Cult*, **47**, 15–26 (1996).

14. Kulkarni, A. A., Kelkar, S., Watve, M. & Krishnamurthy, K. 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 (2007).
15. Thomas, P. *in vitro* decline in plant cultures: detection of a legion of covert bacteria as the cause for degeneration of long-term micropropagated triploid watermelon cultures. *Plant Cell Tiss. Org. Cult.* **77**, 173–179 (2004).
16. Pirttilä, A. M., Podolich, O., Koskimäki, J. J., Hohtola, E. & Hohtola, A. Role of origin and endophyte infection in browning of bud-derived tissue cultures of Scots pine (*Pinus sylvestris* L.). *Plant Cell Tiss. Org. Cult.* **95**, 47–55 (2008).
17. Thomas, P., Swarna, G. K., Patil, P. & Rawal, R. D. 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 Tiss. Org. Cult.* **93**, 39–54 (2008).
18. Ray, S. S., Ali, M. N., Mukherjee, S., Chatterjee, G. & Banerjee, M. Elimination and molecular identification of endophytic bacterial contaminants during *in vitro* propagation of *Bambusa balcooa*. *World J. Microbiol. Biotechnol.* **33**, 1–9 (2017).
19. Anami, S., Njuguna, E., Coussens, G., Aesaert, S. & Van Lijsebettens, M. Higher plant transformation: principles and molecular tools. *Int. J. Dev. Biol.* **57**, 483–494 (2013).
20. Breyer, D., Kopertekh, L. & Reheul, D. Alternatives to antibiotic resistance marker genes for *in vitro* selection of genetically modified plants – scientific developments, current use, operational access and biosafety considerations. *Crit. Rev. Plant Sci.* **33**, 286–330 (2014).
21. Pu, X. & Goodman, R. N. Induction of necrogenesis by *Agrobacterium tumefaciens* on grape explants. *Physiol. Mol. Plant Pathol.* **41**, 241–254 (1992).
22. Deng, W., Pu, X. A., Goodman, R. N., Gordon, M. P. & Nester, E. W. T-DNA genes responsible for inducing a necrotic response on grape vines. *Mol. Plant-Microbe Interact.* **8**, 538–548 (1995).
23. Ozawa, K. Establishment of a high efficiency *Agrobacterium*-mediated transformation system of rice (*Oryza sativa* L.). *Plant Sci.* **176**, 522–527 (2009).
24. Nauerby, B., Billing, K. & Wyndaele, R. Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. *Plant Sci.* **123**, 169–177 (1997).
25. Ogawa, Y. & Mii, M. Screening for highly active β -lactam antibiotics against *Agrobacterium tumefaciens*. *Arch. Microbiol.* **181**, 331–336 (2004).
26. Priya, A. M., Pandian, S. K. & Manikandan, R. The effect of different antibiotics on the elimination of *Agrobacterium* and high frequency *Agrobacterium*-mediated transformation of indica rice (*Oryza sativa* L.). *Czech J. Genet. Plant Breed.* **48**, 120–130 (2012).
27. Grzebelus, E. & Skop, L. Effect of β -lactam antibiotics on plant regeneration in carrot protoplast cultures. *In Vitro Cell. Dev. Biol. Plant*, **50**, 568–575 (2014).
28. Qin, Y. H., Teixeira da Silva, J. A., Bi, J. H., Zhang, S. L. & Hu, G. B. Response of *in vitro* strawberry to antibiotics. *Plant Growth Regul.* **65**, 183–193 (2011).

29. Cheng, Z. M., Schnurr, J. A. & Kapaun, J. A. Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. *Plant Cell Rep*, **17**, 646–649 (1998).
30. Kumar, R., Mamrutha, H. M., Kaur, A. & Grewal, A. Synergistic effect of cefotaxime and timentin to suppress the *Agrobacterium* overgrowth in wheat (*Triticum aestivum* L.) transformation. *Asian J. Microbiol., Biotechnol. Environ. Sci*, **19**, 961–967 (2017).
31. Topping, J. F. & Lindsey, K. Shoot cultures and root cultures of tobacco, in *Plant Tissue Culture Manual*. (ed. K. Lindsey) 67–79 (Springer Netherlands, Dordrecht; 1991)
32. Colgan, R. *et al.* Optimisation of contained *Nicotiana tabacum* cultivation for the production of recombinant protein pharmaceuticals. *Transgenic Res*, **19**, 241–256 (2010).
33. Tremblay, R., Wang, D., Jevnikar, A. M. & Ma, S. Tobacco, a highly efficient green bioreactor for production of therapeutic proteins. *Biotechnol. Adv*, **28**, 214–221 (2010).
34. Moustafa, K., Makhzoum, A. & Tremouillaux-Guiller, J. Molecular farming on rescue of pharma industry for next generations. *Crit. Rev. Biotechnol*, **36**, 840–850 (2016).
35. Zhou, J. *et al.* Characterization of the core microbiome in tobacco leaves during aging. *Microbiologyopen*, **9**, e984–e984 (2020).
36. Chen, X. *et al.* *Nicotiana tabacum* seed endophytic communities share a common core structure and genotype-specific signatures in diverging cultivars. *Comput. Struct. Biotechnol. J*, **18**, 287–295 (2020).
37. Chen, Z. *et al.* Species diversity characteristics of endophytic bacteria in tobacco at different regions of Yunnan Province. *SW. China J. Agric. Sci*, **28**, 857–861 (2015).
38. Wojtania, A., PvBawska, J. & Gabryszewska, E. Identification and elimination of bacterial contaminants from Pelargonium tissue cultures. *J. Fruit Orn. Plant Res*, **13**, 101–108 (2005).
39. Doyle, J. DNA Protocols for Plants, in *Molecular Techniques in Taxonomy*. (eds. G.M. Hewitt, A.W.B. Johnston & J.P.W. Young) 283–293 (Springer Berlin Heidelberg, Berlin, Heidelberg; 1991)
40. Ding, T., Palmer, M. W. & Melcher, U. Community terminal restriction fragment length polymorphisms reveal insights into the diversity and dynamics of leaf endophytic bacteria. *BMC Microbiol*, **13**, 1 (2013).
41. Li, H., Luo, J., Hemphill, J. K., Wang, J. T. & Gould, J. H. A rapid and high yielding DNA miniprep for cotton (*Gossypium* spp.). *Plant Mol. Biol. Rep*, **19**, 183–183 (2001).
42. Wang, H. X., Geng, Z. L., Zeng, Y. & Shen, Y. M. Enriching plant microbiota for a metagenomic library construction. *Environ. Microbiol*, **10**, 2684–2691 (2008).
43. Milani, C. *et al.* Assessing the fecal microbiota: an optimized ion torrent 16S rRNA gene-based analysis protocol. *PLoS One*, **8**, e68739 (2013).
44. Cai, L., Ye, L., Tong, A. H. Y., Lok, S. & Zhang, T. Biased diversity metrics revealed by bacterial 16S pyrotags derived from different primer sets. *PLoS One*, **8**, e53649 (2013).
45. Aloisio, I. *et al.* Evaluation of the effects of intrapartum antibiotic prophylaxis on newborn intestinal microbiota using a sequencing approach targeted to multi hypervariable 16S rDNA regions. *Appl.*

Microbiol. Biotechnol, **100**, 5537–5546 (2016).

46. Winand, R. *et al.* Targeting the 16S rRNA gene for bacterial identification in complex mixed samples: comparative evaluation of second (illumina) and third (oxford nanopore technologies) generation sequencing technologies. *Int. J. Mol. Sci*, **21**, 298 (2020).
47. Torrell, H. *et al.* Multiomic approach to analyze infant gut microbiota: experimental and analytical method optimization. *Biomolecules*, **11**, 999 (2021).
48. Erlacher, A. *et al.* Rhizobiales as functional and endosymbiotic members in the lichen symbiosis of *Lobaria pulmonaria* L. *Front. Microbiol*, **6**, 53 (2015).
49. Abraham, W. R., Rohde, M. & Bennisar, A. The family *Caulobacteraceae*, in *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*. (eds. E. Rosenberg, E.F. DeLong, S. Lory, E. Stackebrandt & F. Thompson) 179–205 (Springer Berlin Heidelberg, Berlin, Heidelberg; 2014)
50. Glaeser, S. P. & Kämpfer, P. The family *Sphingomonadaceae*, in *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*. (eds. E. Rosenberg, E.F. DeLong, S. Lory, E. Stackebrandt & F. Thompson) 641–707 (Springer Berlin Heidelberg, Berlin, Heidelberg; 2014)
51. Velusamy, K. & Krishnani, K. K. Heterotrophic nitrifying and oxygen tolerant denitrifying bacteria from greenwater system of coastal aquaculture. *Appl. Biochem. Biotechnol*, **169**, 1978–1992 (2013).
52. Doronina, N., Kaparullina, E. & Trotsenko, Y. The family *Methylophilaceae*, in *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria*. (eds. E. Rosenberg, E.F. DeLong, S. Lory, E. Stackebrandt & F. Thompson) 869–880 (Springer Berlin Heidelberg, Berlin, Heidelberg; 2014)
53. Torres, A. R. *et al.* Diversity of endophytic enterobacteria associated with different host plants. *J. Microbiol*, **46**, 373–379 (2008).
54. Bünger, W., Jiang, X., Müller, J., Hurek, T. & Reinhold-Hurek, B. Novel cultivated endophytic *Verrucomicrobia* reveal deep-rooting traits of bacteria to associate with plants. *Sci. Rep*, **10**, 8692 (2020).
55. Turenne, C. Y., Tschetter, L., Wolfe, J. & Kabani, A. Necessity of quality-controlled 16S rRNA gene sequence databases: Identifying nontuberculous *Mycobacterium* species. *J. Clin. Microbiol*, **39**, 3637–3648 (2001).
56. Thomas, P., Sekhar, A. C. & Shaik, S. P. High taxonomic diversity of cultivation-recalcitrant endophytic bacteria in grapevine field shoots, their *in vitro* introduction, and unsuspected persistence., **246**, 879–898 (2017).
57. Johnston-Monje, D. & Raizada, M. N. Conservation and diversity of seed associated endophytes in *Zea* across boundaries of evolution, ethnography and ecology. *PLoS One*, **6**, e20396 (2011).
58. Shahzad, R., Khan, A. L., Bilal, S., Asaf, S. & Lee, I. J. What is there in seeds? Vertically transmitted endophytic resources for sustainable improvement in plant growth. *Front. Plant Sci*, **9**, 24 (2018).
59. Fedrizzi, T. *et al.* Genomic characterization of Nontuberculous *Mycobacteria*. *Sci. Rep*, **7**, 45258 (2017).

60. Leff, J. W., Lynch, R. C., Kane, N. C. & Fierer, N. Plant domestication and the assembly of bacterial and fungal communities associated with strains of the common sunflower, *Helianthus annuus*. *New Phytol*, **214**, 412–423 (2017).
61. Wang, W. F., Zhai, Y. Y., Cao, L. X., Tan, H. M. & Zhang, R. D. Illumina-based analysis of core actinobacteriome in roots, stems, and grains of rice. *Microbiol. Res*, **190**, 12–18 (2016).
62. de Vries, H. J. *et al.* Isolation and characterization of Sphingomonadaceae from fouled membranes. *npj Biofilms and Microbiomes*, **5**, 6 (2019).
63. Walsh, C. M., Gebert, M. J., Delgado-Baquerizo, M. & Maestre, F. T. Fierer, N. A global survey of Mycobacterial diversity in soil. *Appl. Environ. Microbiol*, **85**, e01180–01119 (2019).
64. Bouam, A., Armstrong, N., Levasseur, A. & Drancourt, M. *Mycobacterium terramassiliense*, *Mycobacterium rhizamassiliense* and *Mycobacterium numidiamassiliense* sp. nov., three new *Mycobacterium simiae* complex species cultured from plant roots. *Sci. Rep*, **8**, 9309 (2018).
65. Mano, H., Tanaka, F., Nakamura, C., Kaga, H. & Morisaki, H. 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 (2007).
66. Conn, V. M. & Franco, C. M. M. Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by terminal restriction fragment length polymorphism and sequencing of 16S rRNA clones. *Appl. Environ. Microbiol*, **70**, 1787–1794 (2004).
67. Koskimäki, J. J., Hankala, E., Suorsa, M., Nylund, S. & Pirttilä, A. M. Mycobacteria are hidden endophytes in the shoots of rock plant [*Pogonatherum paniceum* (Lam.) Hack.] (Poaceae). *Environ. Microbiol. Rep*, **2**, 619–624 (2010).
68. Pirttilä, A. M., Pospiech, H., Laukkanen, H., Myllylä, R. & Hohtola, A. Seasonal variations in location and population structure of endophytes in buds of Scots pine. *Tree Physiol*, **25**, 289–297 (2005).
69. Kazda, J.. Mycobacteria as habitat microorganisms of sphagnum bog vegetation, in *The Ecology of Mycobacteria*. (ed. J. Kazda)32–36(Springer Netherlands, Dordrecht; 2000)
70. Kazda, J. *et al.* *Mycobacterium cookii* sp. nov. *Int. J. Syst. Bacteriol*, **40**, 217–223 (1990).
71. Quambusch, M., Pirttilä, A. M., Tejesvi, M. V., Winkelmann, T. & Bartsch, M. Endophytic bacteria in plant tissue culture: differences between easy- and difficult-to-propagate *Prunus avium* genotypes. *Tree Physiol*, **34**, 524–533 (2014).
72. Laukkanen, H., Soini, H., Kontunen-Soppela, S., Hohtola, A. & Viljanen, M. A mycobacterium isolated from tissue cultures of mature *Pinus sylvestris* interferes with growth of Scots pine seedlings. *Tree Physiol*, **20**, 915–920 (2000).
73. Zheng, J. Y. *et al.* The effects of tetracycline residues on the microbial community structure of tobacco soil in pot experiment. *Sci. Rep*, **10**, 8804 (2020).
74. Fatahi-Bafghi, M. Antibiotic resistance genes in the *Actinobacteria* phylum. *Eur. J. Clin. Microbiol. Infect. Dis*, **38**, 1599–1624 (2019).

75. Chaïbi, E. B., Sirot, D., Paul, G. & Labia, R. Inhibitor-resistant TEM β -lactamases: phenotypic, genetic and biochemical characteristics. *J. Antimicrob. Chemother*, **43**, 447–458 (1999).
76. Berg, G. *et al.* Plant microbial diversity is suggested as the key to future biocontrol and health trends. *FEMS Microbiol. Ecol*, **93**, fix050 (2017).
77. Sessitsch, A. & Mitter, B. 21st century agriculture: integration of plant microbiomes for improved crop production and food security. *Microb Biotechnol*, **8**, 32–33 (2015).
78. Murashige, T. & Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant*, **15**, 473–497 (1962).
79. Hodges, D. M., DeLong, J. M., Forney, C. F. & Prange, R. K. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds., **207**, 604–611 (1999).
80. Jagendorf, A. T. & Takabe, T. Inducers of glycinebetaine synthesis in barley. *Plant Physiol*, **127**, 1827–1835 (2001).
81. Li, J. H., Wang, E. T., Chen, W. F. & Chen, W. X. Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. *Soil Biol. Biochem*, **40**, 238–246 (2008).
82. Akinsanya, M. A., Goh, J. K., Lim, S. P. & Ting, A. S. Y. Metagenomics study of endophytic bacteria in Aloe vera using next-generation technology. *Genom. Data*, **6**, 159–163 (2015).
83. Tamosiune, I. *et al.* Cold plasma treatment of sunflower seeds modulates plant-associated microbiome and stimulates root and lateral organ growth. *Front. Plant Sci*, **11**, 568924 (2020).
84. Madden, T. L., Tatusov, R. L. & Zhang, J. Applications of network BLAST server. *Methods Enzymol*, **266**, 131–141 (1996).
85. Zakrzewski, M. *et al.* Calypso: a user-friendly web-server for mining and visualizing microbiome-environment interactions., **33**, 782–783 (2017).
86. Paulson, J. N., Stine, O. C., Bravo, H. C. & Pop, M. Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods*, **10**, 1200–1202 (2013).
87. Lex, A., Gehlenborg, N., Strobel, H., Vuillemot, R. & Pfister, H. UpSet: visualization of intersecting sets. *IEEE Trans. Vis. Comput. Graphics*, **20**, 1983–1992 (2014).

Figures

Figure 1

Effect of antibiotic treatment on MDA accumulation in tobacco in vitro shoots. Control (X), 250 mg·L⁻¹ timentin treated (□) and post-antibiotic treatment (Δ) experimental groups were maintained as described in Materials and methods. Data from 4 independent experiments presented as mean ± standard error. The color of the symbols represents significant differences of the mean values compared to the control at each time point (green – $p < 0.05$, red – $p < 0.01$). Different letters denote significant differences among the time points ($p < 0.05$).

Figure 2

Principal coordinate analysis (PCoA) of variability among the bacterial OTU data sets generated for the tobacco shoot control (TC) and post-antibiotic treatment (PA) samples using five 16S rRNA variable region-specific primer pairs. The analysis was carried out using the Bray-Curtis dissimilarity matrix.

Figure 3

Family level OTU association among data sets generated using five 16S rRNA variable region-specific primer pairs.

Figure 4

Family level distribution of reads mapped to high-abundance families generated using five variable 16S rRNA region-specific primer pairs for the control (TC) and post-antibiotic treatment (PA) samples of the tobacco in vitro shoots. Different font color represents classes of the Bacteria domain.

Figure 5

Representative plots of the alpha diversity indices for tobacco in vitro shoot control (TC) and post-antibiotic treatment (PA) microbiome data sets. Cumulative data of all five primer pairs was used to estimate Richness and Chao1 indices, meanwhile data of the V3 region was excluded from the calculation of Evenness and Shannon indices as an outlier.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryinformationTableS3.xls](#)
- [Supplementaryinformation.pdf](#)