1   **Inter- and intracellular colonization of Arabidopsis roots by endophytic actinobacteria and**

2   **the impact of plant hormones on their antimicrobial activity**

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18   antibiotics; electron microscopy.

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22 **ABSTRACT**

23 Many actinobacteria live in close association with eukaryotes like fungi, insects, animals and

24 plants. Plant-associated actinobacteria display (endo)symbiotic, saprophytic or pathogenic

25 life styles, and can make up a substantial part of the endophytic community. Here, we

26 characterised endophytic actinobacteria isolated from root tissue of *Arabidopsis thaliana*

27 (Arabidopsis) plants grown in soil from a natural ecosystem. Many of these actinobacteria

28 belong to the family of *Streptomycetaceae* with *Streptomyces olivochromogenes* and

29 *Streptomyces clavifer* as well represented species. When seeds of Arabidopsis were

30 inoculated with spores of *Streptomyces* strain coa1, which shows high similarity to *S.*

31 *olivochromogenes,* roots were colonised intercellularly and, unexpectedly, also

32 intracellularly. Subsequent exposure of endophytic isolates to plant hormones typically

33 found in root and shoot tissues of Arabidopsis led to altered antibiotic production against

34 *Escherichia coli* and *Bacillus subtilis*. Taken together, our work reveals remarkable

35 colonization patterns of endophytic streptomycetes with specific traits that may allow a

36 competitive advantage inside root tissue.

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38 **INTRODUCTION**

39 Actinobacteria represent a diverse phylum composed of both rod-shaped and filamentous

40 bacteria that can be found in soil, marine and fresh water ecosystems (Goodfellow 2012).

41 The filamentous actinobacteria are versatile producers of bioactive natural products,

42 including two-thirds of all known antibiotics as well as many anticancer, antifungal and

43 immunosuppressive agents (Barka et al. 2016; Bérdy 2005; Hopwood 2007). The

44 actinobacteria are also major producers of industrially relevant enzymes (Anne et al. 1990;

45 van Dissel et al. 2014). Hence, the actinobacteria are of utmost importance for human

46 health, agriculture and biotechnology.

47 The concept of actinobacteria as free-living bacteria has more recently been

48 challenged by studies pointing to their intimate relationships with diverse eukaryotes

49 (Seipke et al. 2012; van der Meij et al. 2017). Indeed, they have been found in association

50 with vertebrates, invertebrates, fungi and plants. Actinobacteria are welcome guests to

51 their hosts due to their ability to produce chemically diverse natural products. Much of the

52 chemical diversity of secondary metabolites produced by actinobacteria has likely evolved

53 because of their interactions with other (micro)organisms in highly diverse environments

54 (Seipke et al. 2012; van der Meij et al. 2017). It is becoming increasingly clear that

55 actinobacteria play a key role in maintaining plant health by contributing to biotic and

56 abiotic stress tolerance (Viaene et al. 2016). For example, actinobacteria produce

57 siderophores for iron acquisition as well as antibacterials and antifungals to protect their

58 host against pathogens (Viaene et al. 2016).

59 Actinobacteria can make up a substantial part of the root endophytic community

60 across the plant kingdom, which is largely determined by an increased relative abundance of

61 the family of *Streptomycetaceae* (Bulgarelli et al. 2012; Edwards et al. 2015; Lundberg et al.

62 2012). The composition of rhizospheric and root endophytic bacterial communities is

63 strongly influenced by soil type (Bulgarelli et al. 2012) as well as by the plant genotype

64 (Perez-Jaramillo et al. 2017). Therefore, part of the microbiome composition of the

65 rhizosphere and endosphere of *Arabidopsis thaliana* grown under controlled conditions in

66 different natural soils is conserved with specific bacterial taxa including members of the

67 actinobacteria (Bulgarelli et al. 2012). Despite their occurrence in the endosphere, very little

68 is known on how most actinobacteria colonise the endosphere and where they reside. An

69 exception is *Streptomyces scabies*, the causal agent of potato scab, which has been studied

70 in detail (Loria et al. 2006; Jourdan et al. 2016; Bignell et al. 2010; Bukhalid et al. 1998).

71 Colonization of root and shoot tissue by actinobacteria is dependent, at least in part,

72 on chemical cues from the plant as was shown for enrichment of actinobacteria by the plant

73 hormone salicylic acid (SA) (Lebeis et al. 2015). SA plays a role in a variety of physiological

74 and biochemical processes and is important as an endogenous signal mediating local and

75 systemic plant defense responses against pathogens and abiotic stress factors (Rivas-San

76 Vicente and Plasencia 2011). SA is detectable in Arabidopsis leaves and roots at

77 concentrations up to 1 µg/g fresh weight (van de Mortel et al. 2012). Hence, endophytic

78 actinobacteria are most likely exposed and responsive to SA and other plant hormones such

79 as jasmonic acid (Halim et al. 2006; Zhao 2010).

80 In this study we isolated endophytic actinobacteria from Arabidopsis roots, grown

81 under controlled conditions as well as from plants grown in an ecological setting.

82 Colonization patterns were monitored and visualised by confocal as well as electron

83 microscopy for *Streptomyces* strain coa1, which shows high similarity with *Streptomyces*

84 *olivochromogenes*, on axencially-grown *A. thaliana*. Finally, we investigated how specific

85 plant hormones affect the antimicrobial activity of endophytic *Streptomyces* isolated from

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86   wild *Arabidopsis* providing a first step towards evaluating the concept of plant-mediated

87   'antibiotic production on demand'.

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90   **RESULTS & DISCUSSION**

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92   **Isolation of endophytic actinobacteria**

93   To confirm the presence of actinobacteria in the endosphere, sterile *A. thaliana* Col-0 plants

94   were grown in a potting soil:sand mixture for two weeks under controlled conditions,

95   followed by harvesting roots and shoot, surface-sterilization and homogenization of the root

96   tissue and plating onto various media selective for actinobacteria (Zhu et al. 2014b). Ten

97   morphologically distinct actinobacterial isolates were obtained. A similar sampling approach

98   was adopted for *A. thaliana* ecotype mossel (Msl) obtained from a natural ecosystem

99   (Mossel, Veluwe, the Netherlands). Next to plating onto selective media, total DNA was

100   extracted in eight replicates from samples obtained from the soil, the rhizosphere, the root

101   endosphere and from a toothpick inserted in the soil. these DNA samples were then

102   analyzed by 16S rDNA-amplicon sequencing. Based on total sequence reads the results of

103   the latter analysis showed that actinobacteria represented on average 22% of the total

104   endophytic population (Fig. 1A). Among the actinobacterial operational taxonomic units

105   (OTUs), the *Streptomycetaceae* and *Micromonosporacea*e were overrepresented.

106   Additionally, 10% the reads of the endophytic population was represented by only two OTUs

107   (137 and 48), belonging to the family of *Streptomycetaceae* (Fig. 2B). This enrichment was

108   not as much observed for the soil, the rhizosphere and bacteria associated with a toothpick,

109   suggesting a species-specific selection among the root endophytic actinobacteria of *A.*

110   *thaliana*. Numbers on total sequence reads and OTUs are listed in Table S1.

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112   Isolation of actinobacteria from the endosphere *A. thaliana* ecotype Msl resulted in 35

113   morphologically different isolates. The 34-465 region of the 16S RNA gene of all isolates was

114   sequenced and the closest hits on the EzBioCloud were confirmed using the MLSA-based

115   phylogeny published recently (Labeda et al. 2017). Linking the OTUs detected by 16S rDNA

116   amplicon sequencing to a single *Streptomyces* species was not always possible due to a

117   relatively low resolution (Girard et al. 2013; Labeda et al. 2017). Nevertheless, the two

118   isolates that were analysed both belonged to the most abundant OTUs *Streptomyces*

119   *clavifer* and *S. olivochromogenes* (Fig. 2A). Out of the 35 isolates, five were closely related to

120   *S. clavifer* and four to *S. olivochromogenes*. Supportive evidence comes from several

121   independent studies, reporting *S. olivochromogenes* from the endosphere of Chinese

122   cabbage roots, potato tubers, medicinal plants and purple henbit (Singh and Gaur 2016; Lee

123   et al. 2008; Doumbou et al. 1998; Kim et al. 2012). Additionally, strains similar to *S. clavifer*

124   were previously isolated from sugarcane, lucerne plants and wheat (Kruasuwan et al. 2017;

125   Franco et al. 2017; Misk and Franco 2011).

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127   Subsequent phenotyping of these endophytic actinobacterial isolates by high resolution

128   imaging by scanning electron microscopy (SEM) revealed spiny spores that are typical of *S.*

129   *clavifer* (Goodfellow 2012), supporting the 16S rDNA-based taxonomic classification of the

130   isolates as *S. clavifer* (Fig. 2B). In contrast, *S. olivochromogenes* poorly develops sporulating

131   mycelium according to Bergey's, which is shown as well for an isolate classified as *S.*

132   *olivochromogenes* (Fig. 2C). However, impaired sporulation was encountered frequently, a

133   phenotype that was dependent on the growth media, which makes this feature non-specific

134   (see next paragraph). The endophytic isolates of *A. thaliana* ecotype Msl showed diverse

135   colony morphologies (for examples see Fig. 3). Remarkably, 20 out of 35 isolates failed to

136   develop on R5 agar plates, suggesting they had either lost key sporulation genes or lacked

137   the ability to develop in the absence of specific nutrients or trace elements. For example, R5

138   agar is known to lack sufficient iron and copper, which is a major reason why *bld* mutants

139   cannot sporulate on this media (Keijser et al. 2000; Lambert et al. 2014). Additionally,

140   several endophytes showed the propensity to open up their colony, with their vegetative

141   mycelium facing upward. This feature is well exemplified by *Streptomyces* sp. MOS31 and

142   MOS18 (Fig. 3H and C). MOS18 is taxonomically related to *S. clavifer* and MOS31 is the only

143   endophytic isolate closely related to *Streptomyces bobili*, a 'neighbour' of *S.*

144   *olivochromogenes* (Labeda et al. 2017). To study the morphological characteristics of MOS31

145   at higher resolution SEM was applied, which revealed a thick sheet of hyphae that turned

146   away from the inside of the colony (Fig. 4A). Additionally, we observed hyphae extending

147   from these sheets, away from the vegetative mycelium (Fig. 4B).

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149   **Endophytic colonization of *A*. thaliana by *Streptomyces***

150   We then wanted to know if and where the isolates enter the root endosphere of *A. thaliana*

151   to get more insight into the yet elusive endophytic biology of *Streptomyces*. Therefore we

152   inoculated spores of *S. olivochromogenes* strain coa1, which was recruited by sterile *A.*

153   *thaliana* Col-0 plants grown in a potting soil:sand mixture, onto sterilised seeds of *A.*

154   *thaliana* Col-0. Attachment of the spores to the seeds was confirmed by SEM (Fig. S1).

155   Seven-day-old seedlings grown from the treated seeds were stained with propidium iodide

156   and subjected to confocal fluorescence microscopy. The results showed that the endophyte

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157 had colonised both leaves and roots (Fig. 5). Strain coa1 attached to the lateral roots in

158 dense pellets, whereas colonization of the leaves involved hyphal growth over the leaf

159 surface. The colonised *Arabidopsis* roots were then fixed for sectioning and high resolution

160 imaging with transmission electron microscopy (TEM). Regions of interest were identified by

161 obtaining 1-μm sections that were stained with toluidine to visualise the bacteria (Fig. 6A).

162 The results showed that coa1 not only colonised the root surface but also the internal root

163 tissue and, remarkably, the intracellular space (Fig. 6BC). No plant cell defects were

164 observed in the imaged samples. Strikingly there is no plant cellular membrane separating

165 *Streptomyces* from the intracellular space.

166 Previous studies showed colonization of the root surface of *Arabidopsis* by actinobacteria,

167 and lettuce, turnip rape and carrot by *Streptomyces* (Bulgarelli et al. 2012; Bonaldi et al.

168 2015; Kortemaa et al. 1994). In addition, endophytic colonization of germinating wheat seed

169 has been reported by reintroduction of a GFP expressing *Streptomyces* strain (Franco et al.

170 2017). To the best of our knowledge, our results show for the first time the presence of a

171 streptomycete within an Arabidopsis root cell. Furthermore, the hyphae of coa1 appeared

172 less electron dense in the endosphere than on the plant surface, which may reflect

173 physiological differences between life inside and outside the plant. Still there are major gaps

174 in our understanding of the endophytic biology of streptomycetes. For example, we did not

175 find reproductive structures or spores within the endosphere. It therefore remains to be

176 seen whether endophytes have a complete lifecycle inside plants or only remain in the

177 vegetative growth phase, although we have to take into account that a seven day timeframe

178 might not be sufficient to form spores within a plant. It has also been suggested that the

179 endophytic lifestyle of actinobacteria may include the formation of small protoplast-like

180 cells that lack a cell wall (Ramijan et al. 2016). These so-called L-forms may explain how the

181   relatively large mycelial actinobacteria can still migrate and proliferate inside plant tissue.

182   To further unravel the endophytic biology of *Streptomyces* we will focus future experiments

183   on the identification of major genetic or morphological traits associated with the endophytic

184   lifestyle.

185

186   **Response of endophytes to phytohormones**

187   Actinobacteria play an important role in antibiosis and as probiotics to the plant due to the

188   production of a diverse array of bioactive molecules (Viaene et al. 2016). We assessed the

189   antimicrobial activity of the actinobacterial endophytes in overlay assays, using *Bacillus*

190   *subtilis* (Gram-positive) and *Escherichia coli* (Gram-negative) as the target. By applying agar

191   diffusion assays with the streptomycetes grown on minimal medium agar with mannitol and

192   glycerol we showed that 11% of the isolates produced one or more compounds that

193   inhibited growth of *E. coli* cells, while 14% inhibited growth of *B. subtilis*. Generally, the vast

194   majority of actinomycetes isolated from soil samples are able to inhibit *B. subtilis* growth

195   under routine laboratory conditions, which differs from what we find for our collection of

196   endophytes. While the small numbers make it difficult to apply statistics, we cannot rule out

197   that the reduced antibiotic activity against Gram-positive bacteria and in particular

198   Firmicutes, may be typical of endophytic actinobacteria.

199

200   Genome sequencing revealed that actinobacteria have a lot of biosynthetic gene clusters for

201   natural products that are poorly expressed (Kolter and van Wezel 2016; Nett et al. 2009).

202   This offers a vast reservoir of potentially important bioactive molecules, including

203   antibiotics. To allow screening of these compounds, strategies are required to elicit their

204   expression (Rutledge and Challis 2015; van Wezel et al. 2009; Wu et al. 2015; Zhu et al.

205   2014a). We assessed the potential of phytohormones as elicitors, thereby mimicking the

206   chemical environment of the rhizosphere and endosphere. For this, we exposed the

207   endophytes to the plant hormones salicylic acid (SA), indole acetic acid (IAA, known as

208   auxin) or jasmonic acid (JA). The strains were grown on minimal media, with or without

209   0.01%, 0.001% or 0.0001% (w/v) of either plant hormone. Square 12x12 cm agar plates were

210   inoculated with spots from spore stocks of the endophytes. Interestingly, the percentage of

211   strains exhibiting antibiotic activity roughly doubled, with 20% of the strains inhibiting *E. coli*

212   cells, and 29% inhibiting growth of *B. subtilis* (Table S1). This effect could mostly be

213   attributed to IAA, which had a more significant eliciting effect on the endophytes than SA or

214   JA (Table S2). An example of elicited antimicrobial production by IAA is presented in Fig. S2.

215   Additionally, we observed increased activity against the indicator strains as well, indicating

216   either more production of the same antibiotic or production of a different (set of)

217   antibiotic(s) (See Fig. S3). Although the concept of eliciting antimicrobial production by

218   actinobacteria is already established, this experimental setup was connected to the biotic

219   interactions between plant and streptomycete, aimed to mimic the chemical environment

220   of the plant. Plant-endophyte interactions have likely played a key role in the evolution of

221   the chemical diversity of actinomycete-derived natural products and signals that control the

222   production of these antimicrobials are likely tied to the biotic interactions. This idea is

223   further explored by the "cry for help" hypothesis, which additionally states that

224   actinobacteria encounter trade-offs between the costs of producing complex natural

225   products and their benefits, and may therefore produce these molecules specifically in

226   response to ecological demands (van der Meij et al. 2017). To broaden our understanding of

227   this concept it would be of interest to know which type(s) of compounds are produced in

228   response to phytohormone exposure. Identification of the antimicrobials produced in

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229   response to phytohormones and metabolic networks will give better insight into the

230   microbes' responses to the plant's "cry for help".

231

232   **CONCLUSION**

233   In this study, we show the recruitment of *Streptomyces* endophytes by *A. thaliana* Col-0

234   and *A. thaliana* Msl. Our pilot study shows that isolates falling within the groups of *S.*

235   *olivochromogenes* and *S. clavifer* are overrepresented in the endosphere, suggesting that

236   these endophytic streptomycetes may have specific characteristics that allow them to adapt

237   to life inside the plant. This needs to be studied in more detail, and may also be extended to

238   the study of other actinobacterial genera found in the endosphere. Additionally, we provide

239   a first step towards the proof of concept for the 'cry for help' hypothesis, whereby plant

240   hormones, in particular IAA, have a stimulating effect on antibiotic production by

241   endophytic actinobacteria. These baseline experiments highlight the importance of

242   exploring and exploiting plant-actinomycete interactions as elicitors for 'antibiotic

243   production on demand'.

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245

246   **MATERIALS AND METHODS**

247

248   **Bacteria, plants and growth conditions**

249   *S. coelicolor* A3(2) M145 was obtained from the John Innes Centre strain collection in

250   Norwich, UK, and *S. griseus* DSM40236 from the DSMZ culture collection (Braunschweig,

251   Germany). *S. coelicolor* M145 and *S. griseus* DSM40236 were grown on SFM for 6 days at 30

252   °C, unless indicated differently. A sterile *A. thaliana* ecotype Columbia (Col-0) was used for

253   the recruitment of endophytic actinobacteria under lab conditions. Plants were grown on a

254   mixture of 9:1 substrate soil and sand (Holland Potgrond) at 21 °C, a 16h photoperiod, and

255   70% relative humidity. After 2 weeks of growth, the plants were harvested. Soil for the field

256   experiment with *A. thaliana* ecotype Mossel (Msl) was collected in May 2016 at the Mossel

257   area at the 'Hoge Veluwe' in the Netherlands (coordinates: N52°03'35.5" E5°45'06.4"), from

258   four different spots within a radius of 100 m. If there was any vegetation present, the top 5-

259   10 cm soil was removed. The soil was homogenised and all large parts such as dead roots

260   and stones were removed. The soil was kept in a cold room at 4 °C until use. Seeds were

261   sterilised in 4x diluted household bleach for 10 min, washed seven times with sterile MQ

262   water, a short rinse with 70% ethanol and transferred to plates with a wet filter paper,

263   placed at 4 °C for 48 hours and then moved to a 21 °C incubator in the dark. Mossel soil was

264   placed in a tray with 3 x 3 cm pots and watered. To remove the endogenous seed

265   population, the tray was placed in the greenhouse for 2 days. After weeding, the sterile

266   seedlings on the plates were transplanted to the tray with Mossel soil and after 7 days the

267   plants including the soil were planted into to the Mossel field. After 6 weeks of growth in

268   the field, plants were harvested using a small shovel 3-4 cm around the base of the plant.

269

Isolation of endophytes

A. thaliana Col-0 was surface sterilised by washing the plant 3 times in 70% EtOH, after which the plant material was crushed in MQ. Sterilization was confirmed by adding a sterilised plant onto LB agar, after which no bacterial growth was observed. Root tissue of *A. thaliana* Msl was cleaned, sonicated and ground with mortar and pestle in 1 mL phosphate buffer (per litre: 6.33 g of $NaH_2PO_4 \cdot H_2O$, 10.96 g of $Na_2HPO_4 \cdot 2H_2O$ and 200 µL Silwet L-77). Both the crushed plant material and EC prep were spread onto the surface of a range of selective isolation media including humic acid agar (HA) (Hayakawa and Nonomura 1987), glucose casein agar (GCA) (Zhang 1985), soy flour mannitol medium (SFM) or minimal medium (MM) (Kieser et al. 2000). Initial selection was done on media supplemented with the antifungal agent nystatin (50 µg/ml) and the antibacterial agent nalidixic acid (10 µg/ml). Plates were incubated at 30°C for 4 to 25 days.

Plant harvesting and DNA isolation of the microbial community

For *A. thaliana* Msl we applied the harvesting protocol as described before (Lundberg et al. 2012). In short, roots including rhizospheric soil were collected in a 50 mL tube containing 25 mL of phosphate buffer, and vortexed for 15 seconds. Replacing the buffer and vortexing was repeated until the buffer stayed clear. Roots were transferred to a 15 mL tube, sonicated (5 bursts of 30 seconds with 30 seconds breaks), vortexed, washed with phosphate buffer and dried on filter paper. Then, the roots were either ground for bacterial isolation, or flash frozen and stored at -80 °C for later DNA isolation. Four individual plants were pooled into one sample. DNA was isolated from soil and EC samples using the MoBio PowerSoil kit and the MP Bio Fast DNA spin kit, respectively. Quality and quantity of the

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293   DNA was checked by nanodrop and gel electrophoresis. Around 400 ng was sent for 16s

294   rDNA sequencing at Beijing Genomics Institute (BGI).

295

296   **Amplicon sequencing**

297   Using primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNVGGGTWTCTAAT-3'

298   ), the V4 region was sequenced at BGI on the HiSeq2500 sequencing platform (Illumina). Raw data

299   from BGI was processed using a previously reported custom implementation (Perez-

300   Jaramillo et al. 2017) of QIIME (Caporaso et al. 2010) with minor modifications (described by

301   Schneijderberg et al., in prep). In short, reads were quality filtered and filtered for chimeras

302   using ChimeraSlayer. Using a 97% identity threshold, *de novo* OTUs were determined, which

303   were taxonomically assigned using the RDP classifier 2.10 (Cole et al. 2014) with the

304   GreenGenes database 28 (DeSantis et al. 2006). OTUs related to mitochondial and

305   chloroplast sequences were removed, as were the OTUs that did not have 25 reads in at

306   least 5 samples ("rare taxa"). To obtain relative abundance of the OTUs, the number of

307   reads from a single OTU per sample was divided by the total number of reads of that sample

308   after filtering for rare taxa.

309

310   **Analysis of actinobacteria based on 16S rRNA sequences**

311   The 16S rRNA genes of the actinobacteria were amplified by PCR from liquid-grown mycelia

312   using primers F1 (5'-GCGTGCTTAACACATGCAAG-3') and R1 (5'-CGTATTACCGCGGCTGC T G-

313   3'), which correspond to nt positions 15-34 and 465-483 of the 16S rRNA locus of *S.*

314   *coelicolor* (van Wezel et al. 1991), respectively. PCRs were conducted as described (Colson

315   et al. 2007) and sequenced using oligonucleotide F1. Sequencing was done at BaseClear in

316   Leiden, the Netherlands. 16S rRNA gene analysis was performed using web based identify

317   tool on EzBioCloud (https://www.ezbiocloud.net/identify). The identify service provides

318   proven similarity-based searches against quality-controlled databases of 16S rRNA

319   sequences. The top-hit information for each Identify Job was checked against *Streptomyces*

320   focused MLSA based phylogenetic tree published elsewhere (Labeda et al. 2017).

321

322   **Microscopy**

323   *Light microscopy*

324   Stereo microscopy was done using a Zeiss Lumar V12 microscope equipped with a AxioCam

325   MRc, and confocal microscopy using a Zeiss Observer Microscope. For confocal microscopy,

326   spores of the endophyte were added to the seeds. Seeds were put on ½ Murashige & Skoog

327   medium with 1% sucrose and 0.8% agar and kept in the dark at 4 degrees for 48 hours. After

328   the cold shock, seeds were incubated in the climate room for 1 week after which they were

329   imaged. Samples were stained with propidium iodide 1:1000 (1 µg/ml). Samples were

330   excited with laser light at a wavelength 535 nm to detect the propidium iodide.

331

332   *Electron microscopy*

333   Morphological studies on single colonies of endophytes by SEM were performed using a

334   JEOL JSM6700F scanning electron microscope. For Streptomycete only samples, pieces of

335   agar with biomass from 6-day-old colonies grown on SFM were cut and fixed with 1.5%

336   glutaraldehyde (1 hour). Subsequently, samples were dehydrated (70% acetone 15 min, 80%

337   acetone 15 min, 90% acetone 15 min, 100% acetone 15 min and critical point dried (Baltec

338   CPD-030). Hereafter the samples were coated with gold using a gold sputter coater, and

339   directly imaged using a JEOL JSM6700F. For SEM of Arabidopis samples, imaging timing was

340   increased four-fold to optimise fixation and dehydration, and the 70% acetone step was

341   done overnight.

342   Transmission electron microscopy (TEM) for the analysis of cross-sections of *Arabidopsis*

343   roots and *Streptomyces* was performed with a JEOL1010 transmission electron microscope

344   as described previously (Piette et al. 2005).

345   Samples were grown in the same way as for the light microscopy, and fixed with 1.5%

346   glutaraldehyde for 4 hours. Post-fixation was performed with 1% Osmium tetroxide for 4

347   hours. Initial dehydration with 70% ethanol was done overnight. Hereafter using a high

348   magnification (150x) stereo microscope (MZ16AF) root sections containing *Streptomyces*

349   growth were selected, followed by dehydration in 1 hr steps (80%, 90%, 100% ethanol,

350   100% propylene oxide, 50/50 propylene oxide/EPON, 100% EPON). Subsequently samples

351   were embedded in EPON and polymerised for 2 days at 60° Celsius. Ultrathin sections were

352   cut on an ultramicrotome (Reichert Ultracut E), collected on copper grids and examined

353   using a Jeol1010 transmission electron microscope at 70 kV.

354

355   **Antimicrobial activity assays**

356   Antimicrobial activity of the endophytes was tested against *Bacillus subtilis* 168 or a

357   derivative of Escherichia coli AS19-RrmA (Liu and Douthwaite 2002). Indicator bacteria were

358   cultured in LB broth and incubated at 37 °C overnight. Antimicrobial assays were conducted

359   using the double-layer agar method. Briefly, actinobacteria were inoculated on minimal

360   medium agar plates containing mannitol and glycerol as the carbon sources (1% w/v),

361   supplemented with either (+-)-jasmonic acid (Cayman chemical company, cas: 88-30-0), 3-

362   indoleacetic acid (Sigma-Aldrich, cas: 87-51-4) or salycyclic acid (Alfa Aesar, cas: 69-72-7).

363   The endophytes were typically incubated for 5 days at 30 °C, following which they were

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364   overlaid with LB soft agar (0.6% w/v agar) containing 300 µl of one of the indicator strains

365   (OD 0.4 - 0.6), and then incubated overnight at 37 °C. The following day, antibacterial

366   activity was determined by measuring the inhibition zones (mm) of the indicator strain

367   surrounding the colonies.

368

369   **Acknowledgements**

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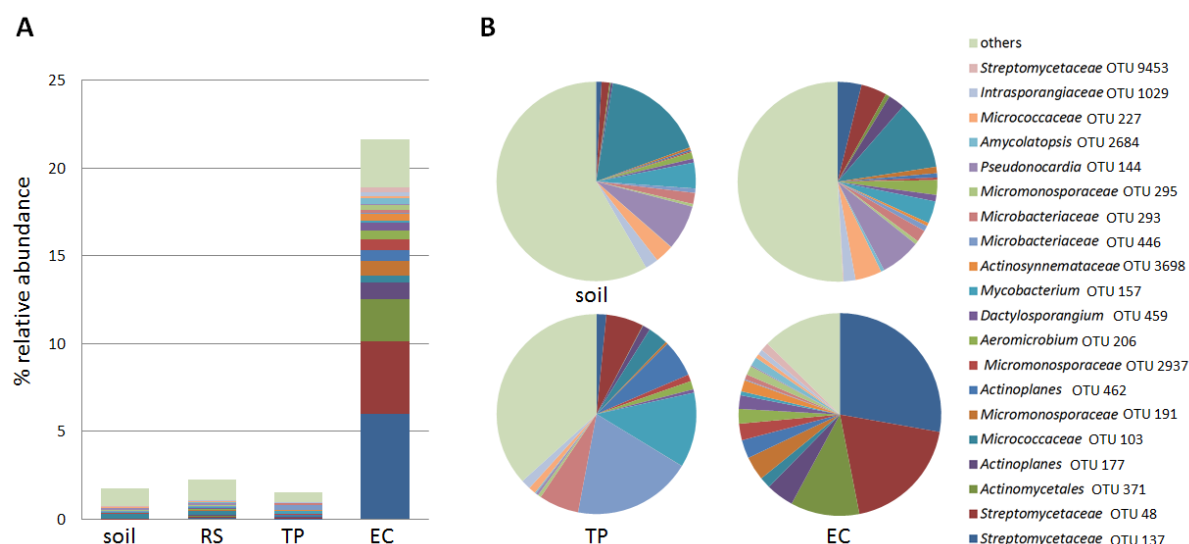507   isolated from mountain soils. Microbiology 160:1714-1725.
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Figure 1. Relative abundance of the actinobacteria in the endophytic compartment of *A.*

***thaliana* Msl.** A) Amplicon sequencing data show that actinobacteria represent 22 % of the

total endophytic compartment (EC), while in soil and rhizosphere (RS) communities and

those in association with a toothpick (TP) inserted in the soil, they take up roughly 2 % of

the total bacterial community. B) The enrichment of actinobacteria is predominately driven

by *Streptomycetaceae* OTU 48 and 137. OTU 48 and 137 make up 10% of the total EC and

almost 50% of all the actinobacteria present in the EC. These OTUs are not as much enriched

in the samples derived from non-endophytic origins.

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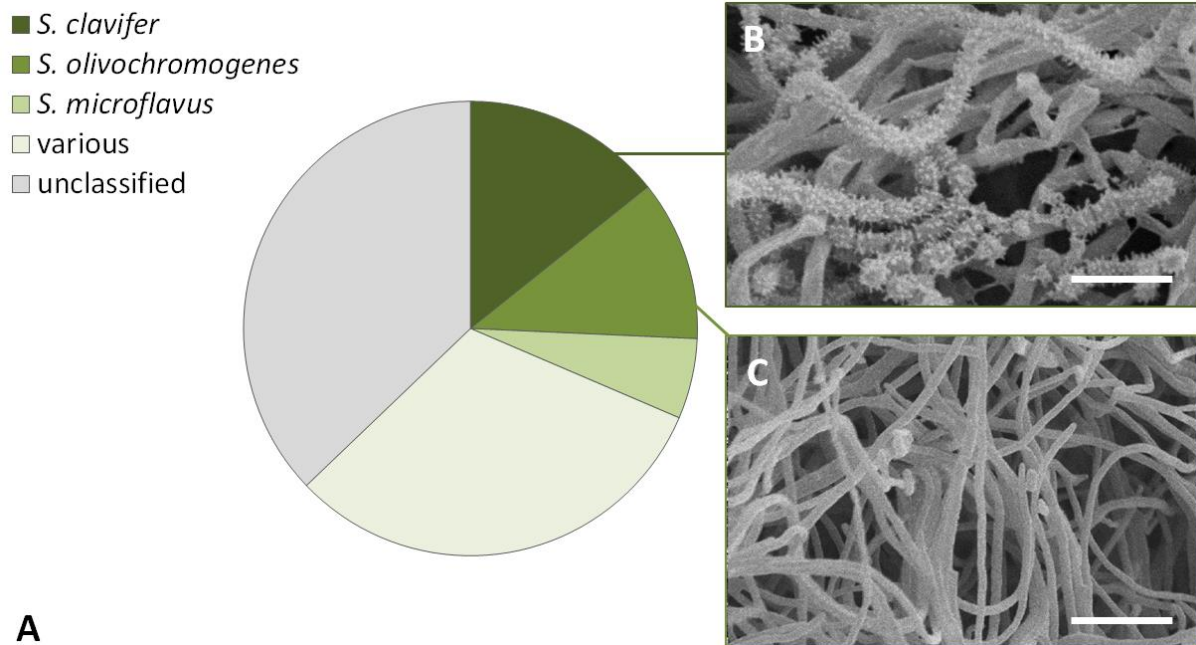
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Figure 2. Characterization of *Streptomyces* endophytes and their taxonomic distribution.

A) Several isolates show highest similarity with *Streptomyces clavifer* or *Streptomyces olivochromogenes* on the basis of 16S rDNA analysis. The majority of the isolates showed a wide variation in closest species assigned, indicating a diverse endophytic compartment. 'Unclassified' means that these actinobacteria could not be classified at the species level based on the 16S rDNA sequence. B) Scanning electron micrograph of *Streptomyces* sp. MOS18, which produces spiny spores. Scale bar: 3 µM. C) Scanning electron micrograph of *Streptomyces* sp. MOS38 showing poor sporulation. Scale bar: 5 µM. Strains were grown on SFM media for 6 days.
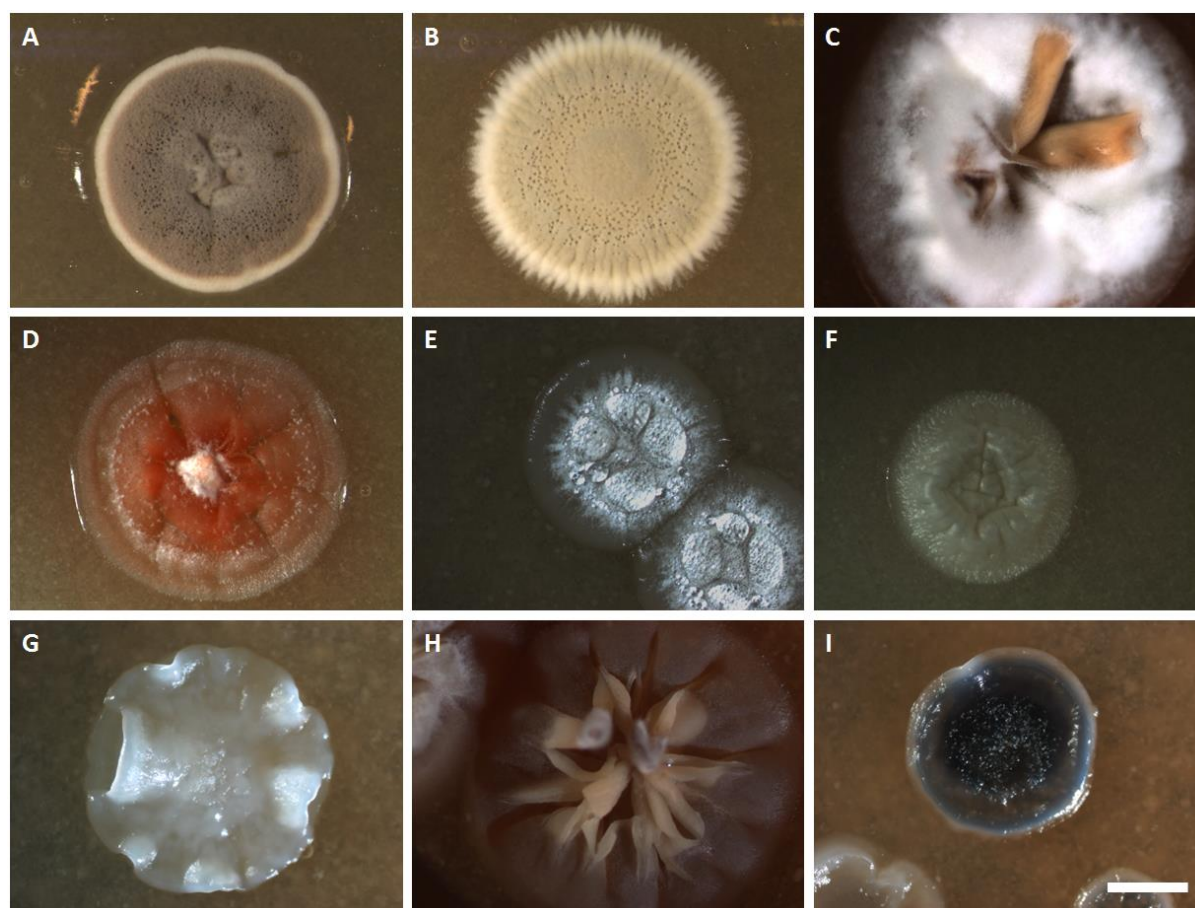
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Figure 3. *Streptomyces* endophytes display a wide range of morphologies. Strains shown above are grown on SFM agar plates for 6 days. *S. coelicolor* M145 and *S. griseus* DSM40236 are shown as reference strains. A) *S. coelicolor* M145 B) *S. griseus* DSM40236 and the endophytic streptomycetes MOS18 (C), MOS38 (D), MOS14 (E), MOS32 (F), MOS25 (G), MOS31 (H) and MOS35 (I). Scale bar: 2 mm.
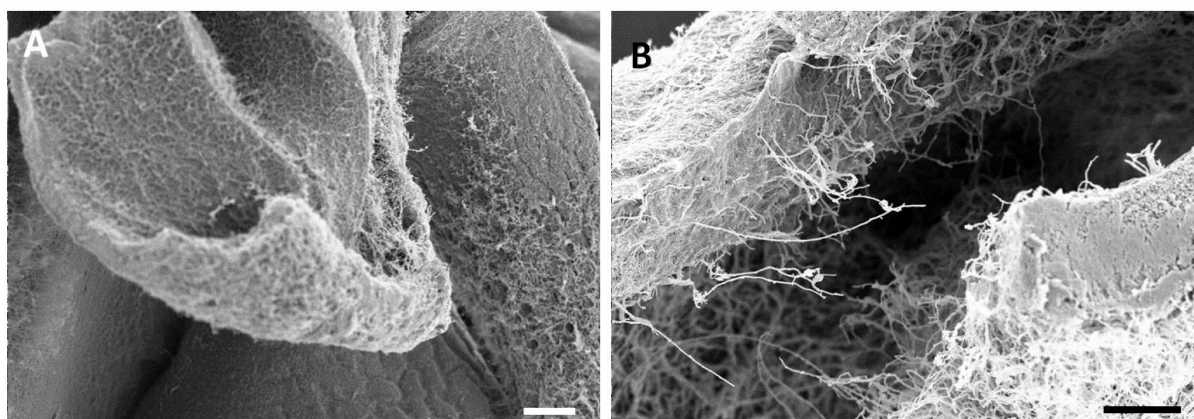
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Figure 4. Scanning electron micrograph of *Streptomyces* sp. MOS31. A) Thick layers of vegetative mycelium made up of hyphae and extracellular matrix turn away from the inner part of the colony. Scale bar: 100 µM. B) Hyphae extending from the mycelial sheets, thereby growing away from the vegetative mycelium. Scale bar: 30 µM. *Streptomyces* sp. MOS31 was grown on SFM agar plates for 6 days.
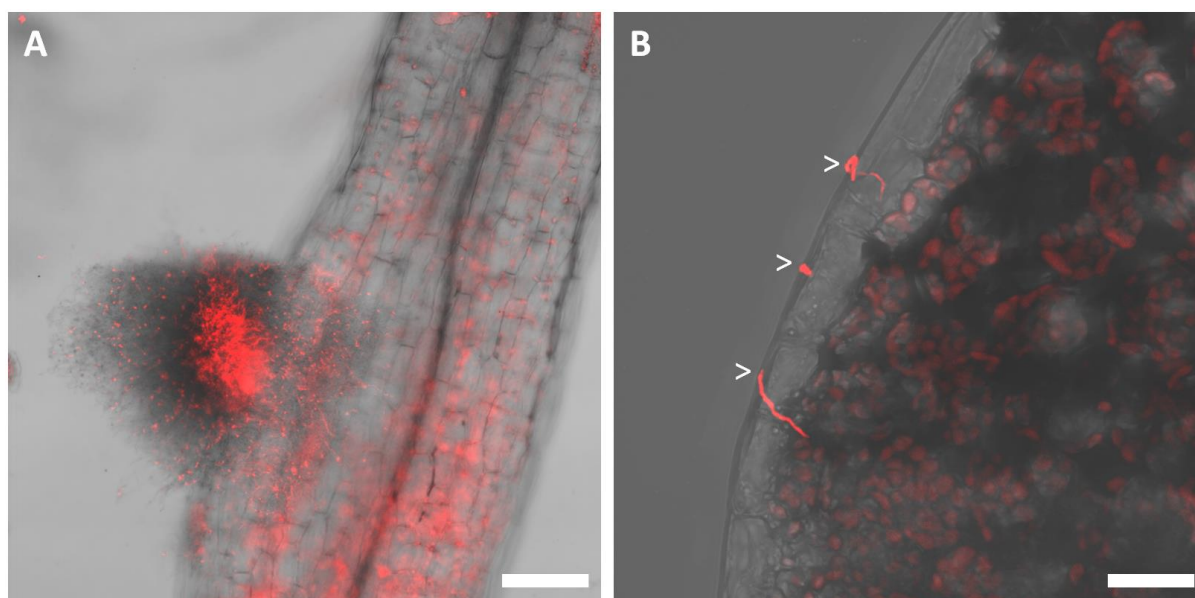
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Figure 5: Colonization of *Arabidopsis* by *Streptomyces* endophyte coa1. A) Confocal micrograph of a colonised lateral root. The sample is stained with propidium iodide, resulting in red fluorescence of both bacterial and plant cells. Coa1 attaches to the root as a dense pellet. Scale bar: 50 μM. B) Confocal micrograph of the border of the leaf. Single hyphae are growing over the leaf surface (arrowheads). Scale bar: 15 μM.
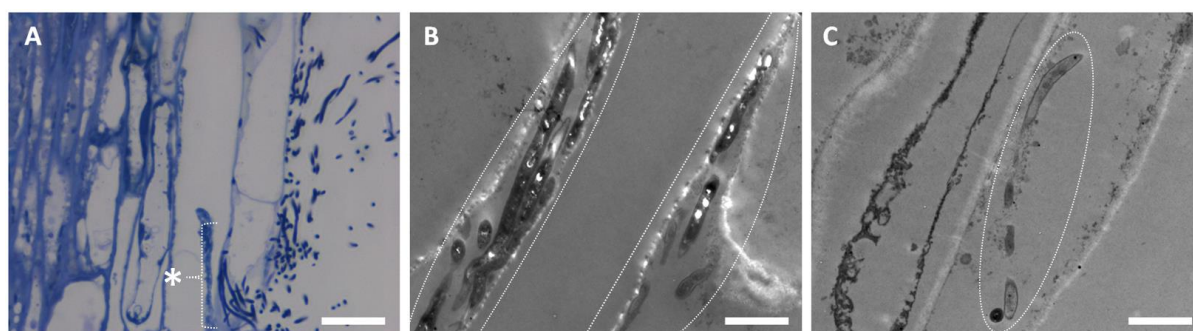
Figure 6: Sections of *Arabidopsis* roots invaded by *Streptomyces* coa1. A) Toluidine stained section of an *Arabidopsis* root invaded by *Streptomyces* coa1. The bacterium enters the root via intercellular space (asterisk). Scale bar: 10 µM. B & C) transmission electron micrographs of *Arabidopsis* roots. Image B shows intercellular presence of the bacterium (between dashed lines) whereas image C shows intracellular growth of *Streptomyces* coa1 in *Arabidopsis* root cells (oval). Scale bar: 2 µM.