Visualization of grapevine root colonization by the Saharan soil isolate Saccharothrix algeriensis NRRL B-24137 using DOPE-FISH microscopy

Stéphane Compant, Saima Muzammil, Ahmed Lebrihi, Florence Mathieu

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Abstract
Background and aim There is currently a gap of knowledge regarding whether some beneficial bacteria isolated from desert soils can colonize epi- and endophytically plants of temperate regions. In this study, the early steps of the colonization process of one of these bacteria, *Saccharothrix algeriensis* NRRL B-24137, was studied on grapevine roots to determine if this beneficial strain can colonize a non-natural host plant. An improved method of fluorescence in situ hybridization (FISH), the double labeling of oligonucleotide probes (DOPE)-FISH technique was used to visualize the colonization behavior of such bacteria as well as to determine if the method could be used to track microbes on and inside plants.
Methods A probe specific to *Saccharothrix* spp. was firstly designed. Visualization of the colonization behavior of *S. algeriensis* NRRL B-24137 on and inside roots of grapevine plants was then carried out with DOPE-FISH microscopy.
Results The results showed that 10 days after inoculation, the strain could colonize the root hair zone, root elongation zone, as well as root emergence sites by establishing different forms of bacterial structures as revealed by the DOPE-FISH technique. Further observations showed that the strain could be also endophytic inside the endorhiza of grapevine plants.
Conclusions Taking into account the natural niches of this beneficial strain, this study exemplifies that, in spite of its isolation from desert soil, the strain can establish populations as well as subpopulations on and inside grapevine plants and that the DOPE-FISH tool can allow to detect it.

Keywords *Saccharothrix algeriensis* NRRL B-24137 · Grapevine · Colonization · DOPE-FISH

S. Compant · S. Muzammil · A. Lebrihi · F. Mathieu
LGC UMR 5503 (CNRS/INPT/UPS), Département Bioprocédés et Systèmes Microbiens, ENSAT-INP de Toulouse, Université de Toulouse,
1 Avenue de l'Agrobiopôle, B.P. 32607,
31326 Castanet-Tolosan Cedex 1, France

A. Lebrihi
Université Moulay Ismail,
Marjane II, BP 298, Meknès, Morocco

Present Address:
S. Compant (✉)
Bioresources Unit, Health & Environment Department,
AIT Austrian Institute of Technology GmbH,
3430 Tulln, Austria
e-mail: stephane.compant@ait.ac.at

Introduction

Plant growth-promoting bacteria as well as biocontrol bacteria could be isolated from the phyllosphere, anthosphere, carposphere, as well as the caulosphere. The majority of these bacterial microsymbionts are,

however, epiphytics and colonize the rhizosphere (Lugtenberg and Kamilova 2009). A subset of the rhizosphere microflora can also enter inside plants, establishing subpopulations, and proliferate as endophytes. This has been well described with cultivable approaches as well as with metagenomic and microscopy analysis (Rosenblueth and Martínez-Romero 2006; Hallmann and Berg 2007; Compant et al. 2010a; Reinhold-Hurek and Hurek 2011). Currently, there is a gap in knowledge regarding plant growth-promoting agents or biocontrol strains colonizing the rhizosphere as well as the endosphere of plants. Particularly, some microbes could be isolated from harsh environments such as desert soils (Compant et al. 2010b), and could act as plant growth-promoting bacteria or biocontrol agents once applied on a crop. Harsh environments might provide a rich source of beneficial bacteria, and it is becoming increasingly evident that microbes from soil and plants growing in harsh environments, such as desert soil, may represent an enormous untapped genetic reservoir for plant improvement. It has been recently postulated that transferring these microbes from native plants to non-host plants promises a revolutionary biotechnology to rapidly improve plant germplasms (Barrow et al. 2008). However, the microbial colonization of such strains on non-host plants should be studied, and also visualization of the process of colonization should be done if any application is carried out on crops growing in temperate conditions (Compant et al. 2010b). Additionally, visualization of plant colonization by these bacteria could allow to better understand niches of colonization of plant-associated bacteria.

In correlation to the research of beneficial microbes from harsh environments, such as desert soils, an actinomycete member of the Actinosynnemataceae family was isolated from a desert soil in a palm grove of Adrar in Algeria. The strain was identified as *Saccharothrix algeriensis* NRRL B-24137 (Zitouni 1995; Zitouni et al. 2004). The strain NRRL B-24137 is known as secreting various secondary metabolites, such as dithiolopyrrolones, with broad bioactive activities (Lamari et al. 2002a, b; Zitouni et al. 2005). The strain can also act as a biocontrol agent. It reduces *Botrytis cinerea* infection on grapevine plants under greenhouse at 25 °C (Muzammil 2012) but also even under high temperature conditions (Muzammil et al. 2011). However, colonization processes of this strain should be studied to understand where the beneficial strain could be localized following soil application to know its niches of colonization. Moreover, this needs to be determined before to study mechanisms of plant resistance responsible of reduction of phytopathogen infections (postulate described by van Loon et al. (1998)).

To visualize colonization and to track microbes on and inside plants various tools, such as *gfp*, *dsred*, and *gus* markers as well as derivatives, could be used (Larrainzar et al. 2005). However, this implies that the microbe will be transformed before application, which is difficult to achieve for some microorganisms. Other methods were used to monitor the colonization behavior of bacteria, such as the DNA-intercalating stains acridine orange, SYTO® dyes, 4′,6-diamidino-2-phenylindole, protein, or lipid stains. However, to be sure to visualize specific taxa of bacteria on and or inside plants, immunostaining with fluorescent-labeled antibodies or fluorescence in situ hybridization (FISH) method (Amann et al. 1990; Wagner et al. 2003) should be used. They can, indeed, provide high specificity to detect given bacterial species or strains (Brandl and Monier 2005; Brandl 2009). For one of these techniques, FISH, visualization can however suffer from some limited advantages for some microorganisms (Wagner et al. 2003). Different improvements have been published then to increase the signal in FISH (Wagner and Haider 2012). In 2010, Stoecker et al. described, for instance, the use of double labeling of oligonucleotide probes (DOPE)-FISH corresponding to 5′- and 3′-double-labeled probes instead of single-labeled probes for FISH. It has been demonstrated that double-labeled probes strongly increase in situ accessibility of rRNA target sites, and increase fluorescent signal. Moreover, this technique provides more flexibility for probe design (Stoecker et al. 2010) and can allow visualizing microorganisms that could not be well detected by single FISH (due to low fluorescent signal) or that are difficult to transform, such as some filamentous microbes.

In this study, we created a specific probe for *Saccharothrix* spp., and we used DOPE-FISH technique to monitor the early colonization process of the beneficial strain NRRL B-24137 on grapevine plants, both in the rhizosphere and in the root endosphere of plants. This was a prerequisite to better understand the interaction between a bacterium isolated from a harsh environment, such as from a Saharan soil, and a non-natural host.

Materials and methods

Bacterial culture

S. algeriensis NRRL B-24137 was used throughout this work. This strain was grown at 30 °C on ISP 2 (International *Streptomyces* Project 2) solid medium (pH 7.0) containing per liter of distilled water: 4 g D(+) glucose (Acros organics), 10 g malt extract (Fluka), 4 g yeast extract (Fluka), and 18 g agar (Sigma). Eight days after growing on plate, aerial mycelium+spores of strain NRRL B-24137 were harvested in phosphate buffered saline (PBS) and concentration was adjusted to 5.10^7 CFU mL^{-1}.

Plant material

Grapevine plants, harboring as a graft part *Vitis vinifera* L. cv. Cabernet Sauvignon clone 15 and 44–53 M (*Vitis riparia* × Malègue) as a rootstock were provided by "Pépinières Colombie Vendries" (Camparnaud, France). These grapevine plants were used because it has been previously described that the strain NRRL B-24137 could be beneficial once applied on them and reduce *B. cinerea* infection (Muzammil et al. 2011). These grapevine plants were stored at 4 °C in a dark cold chamber for at least 2 weeks before being treated with 0.05 % cryptonol for 15 h at ambient temperature (20–25 °C). Plants were then surface sterilized with 1.6 % bleach (10 min) and 70 % ethanol (30 min) before rinsing with sterile tap water. Then, they were planted in two times autoclaved soil containing 1/3 perlite, 1/3 potting soil, and 1/3 sand. Plants were allowed to grow in a phytotronic growth chamber (16-h photoperiod, 20–25 °C night–day, and 70 % relative humidity) and watered with sterile tap water.

Plant inoculation

One month after planting, grapevine plants were delicately separated from their soils. The root systems were dipped in a bacterial solution of *S. algeriensis* NRRL B-24137 (or PBS) for 3 min before plants are placed in pots filled with soils (as described before). Plants were then allowed to grow for 10 days (time allowing resistance to *B. cinerea* described in Muzammil (2012)) before sampling of the plant parts for microscopic analysis.

Probe design for *Saccharothrix* spp. and labeling

To create probes specifics to *Saccharothrix* spp., the partial 16S rRNA gene sequence of *S. algeriensis* NRRL B-24137 (accession: AY054972.2 GI: 134034183) was used. The design of 16S rRNA probes was made by using the Stellaris™ FISH Probes software. The specificity of probes created was then checked on National Center for Biotechnology Information (NCBI), Silva, Green genes blast (Altschul et al. 1997) or on Probe Check at microbial-ecology.net (Loy et al. 2007). ΔG, FA, FAm, as well as the hybridization efficiency were calculated according to Yilmaz and Noguera (2004), (2007), and Yilmaz et al. (2006) and the Tm was calculated by using $Tm = 64.9 + 41 \times ((G + C - 16.4)/\text{length})$ according to Loy et al. (2007). These parameters were evaluated for different temperatures of hybridization.

Among all probes designed, one found as specific to *Saccharothrix* spp. was then purchased at Genecust (Luxemburg) with an aminomodifier C6 at 5′ and 3′ positions (for DOPE-FISH). The probe was then labeled with dylight488 fluorochrome (Piercenet) enabling green fluorescence under UV light.

DOPE-FISH microscopy

To visualize the rhizosphere/rhizoplane colonization by the strain NRRL B-24137, roots of grapevine were cut in small parts 10 days post-inoculation with the strain NRRL B-24137. The samples were then fixed overnight at 4 °C in a paraformaldehyde solution (4 % in PBS) in Eppendorf tubes. The samples were rinsed twice with PBS before to be treated with 1 mg/mL lysozyme at 37 °C during 15 min. They were then rinsed with PBS and dehydrated in an ethanol series (50–99.9 %; 30 min for each step). The DOPE-fluorescence in situ hybridization was then carried out according to Compant et al. (2011) by using 15 ng/μL of a probe specific to *Saccharothrix* spp., labeled at both 5′ and 3′ with the DyLight 488 fluorochrome. Following a DOPE-FISH hybridization at 51 °C, a posthybridization step was carried out at 52 °C and samples were rinsed with sterile distillated water (prewarmed at 51 °C). The samples were then kept in dark during at least 1 day. The samples were then observed using epifluorescence microscope (BH2, Olympus, Japan) under a UV light and pictures were taken with a camera (TCC-3.3ICE-N, Tucsen, China).

In parallel to the rhizosphere colonization study, a possibility of endophytism was evaluated for *S. algeriensis* NRRL B-24137. For this, root samples were treated as described before, except that after the ethanol series, the samples were included in LR white resin according to the manufacturer instructions. Embedded tissues were then sliced with a microtome and glass knives. Slices of 1–1.5 μm were deposited on microscopic slides previously treated with 70 % ethanol. The DOPE-FISH was then carried out as described before. Following the steps of DOPE-FISH hybridization, posthybridization, and rinsing, the slides containing slices were kept in dark during at least 1 day. Slices on slides were then observed using epifluorescence microscope (BH2, Olympus, Japan) under UV light and pictures were taken, as described before. All experiments have been repeated on three independent times with similar results on ten plants each time. More than 20 slices were used per plant to visualize the colonization process.

Results

Design of a probe specific to four species of *Saccharothrix*

Different probes with lengths of 20 and 25 nt were designed. However, no probe with these lengths was specific to *Saccharothrix* spp. Therefore, additional probes of 30 nt were created. Among them, a probe named Sac135 (Table 1) was designed and checked on probe check, Silva, green genes, and NCBI databases. Data revealed that the probe is specific to four species of *Saccharothrix* including *S. algeriensis*. The probe has a Percent G-C content of 53.3, is at position 135 according to the *Escherichia coli* gene numbering, has an exp Td (Tm) of 64 °C; a ΔG_1, −33.0 kcal/mol; a ΔG_2, 0.1 kcal/mol; a ΔG_3, −14.4 kcal/mol; a $\Delta G_{\text{overall}}$, −18.2 kcal/mol; and a FAm of 47.3 % at 51 °C of hybridization with 0.9M Na^+ (Table 1). At 46 °C which is used for the majority of FISH experiments, this probe could not be used due to an inefficient hybridization and a high Fam according to Yilmaz and Noguera (2004, 2007) and Yilmaz et al. (2006). This probe was then used with a formamide concentration of 20 % as hybridization efficiency at 0–20 % of formamide was of 1.0000 (Table 1).

Table 1 The probe and specificity of the probe related to *Saccharothrix* spp

Probe name	Sac135
Target molecule	16S rRNA
Sequence	5′-TAG TTT CCC AGG CTT ATC CCG GAG TAC AGG-3′
Specifity	*S. algeriensis*, *S. espanaensis*, *S. australiensis*, *S. yanglingensis*
Length nt	30
% GC content	53.3
ΔG [kcal/mol]	ΔG_1: −33.0 ΔG_2: 0.1 ΔG_3: −14.4 $\Delta G_{\text{overall:}}$ −18.2
Position[a]	135–165
Tm[b] [°C]	64 °C
MW [g/mol]	9198.02
Formamide %	0–20 %
Hybridation efficiency[c]	1.0000
FAm[d] %	47.3

[a] Probe position according to the *E. coli* gene numbering

[b] Melting temperature dissociation

[c] Hybridation efficiency at 0 % of formamide

[d] Melting formamide concentration (%)

ΔG, formamide percent, hybridation efficiency, and FAm percent calculated for 51 °C and 0.9M Na^+

Grapevine root surface colonization by *S. algeriensis* NRRL B-24137

Colonization of grapevine plants by the strain NRRL B-24137 was evaluated by DOPE-FISH analysis using the Sac135 probe. Grapevine colonization by strain NRRL B-24137 was firstly evaluated at the root hair zone level. Without hybridization with the probe, no green fluorescence signal was recorded under the UV wavelength (data not shown). After hybridization, the bacterium was visualized as spores or as germinated spores, in close contact to the surface of root hairs (Fig. 1a). Hyphae were also visualized as colonizing externally the basis of root hairs (Fig. 1b). Packs (or pellets) of hyphae as well were additionally visualized at the root hair level (Fig. 1c–d). Although some bacteria were isolated on the surface of the root hair zone, in some other few parts some rhizodermal cells were completely filled with bacteria. This was demonstrated with strong green fluorescence signal (Fig. 1f), whereas root cell fluorescence was normally low yellow autofluorescent on roots of plants treated with NRRL B-24137 (Fig. 1e) or control treatment (data not shown).

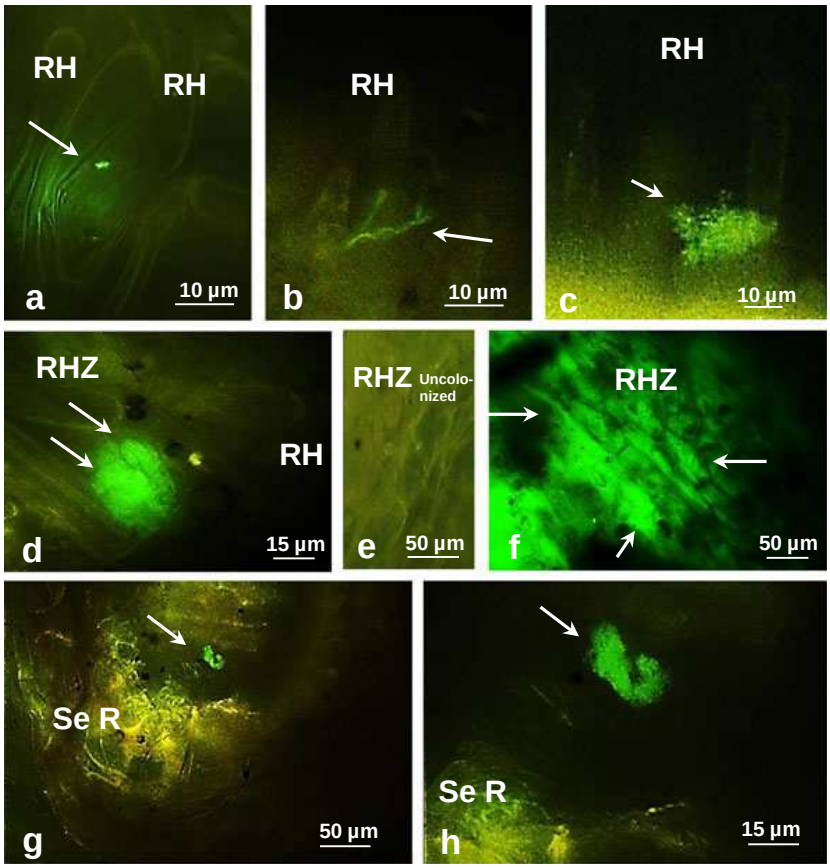

Fig. 1 Microphotographies of the grapevine root colonization by *S. algeriensis* NRRL B-24137 by DOPE-FISH microscopy showing (*arrows*) spores or germinated spores (**a**), hyphae (**b**), a pack (or pellet) of hyphae (**c**–**d**) colonizing root hairs and some rhizodermal cells not filled (**e**) or completely filled (**f**) at the root hair level as well as hyphae at root emergence sites (**g**–**h**). *RHZ* root hair zone, *RH* root hair, *SeR* secondary root

Ten days post-inoculation, the bacterium was also found at root emergence sites as hyphae (Fig. 1g–h). No spores, or germinated spores, were however visualized. Only this type of actinobacterial form was noticed in all analyzed samples.

Colonization by the strain NRRL B-24137 was evaluated not only at the root hair zone and emergence site levels but also at the root elongation zone. The bacterium was visualized as spores or germinated spores (Fig. 2a–b) on the rhizoplane. Visualization of the process of colonization revealed that the bacterium could be also detected as hyphae near some cells of the rhizodermis (Fig. 2c). Colonization of parts of some rhizodermal cells (Fig. 2d) but also the whole outline of some cells on the rhizoplane was noticed as filled by bacteria (Fig. 2e). In contrast to the root hair zone, root emergence site and root elongation zone, no detection of the strain NRRL B-24137 was reported on any of the analyzed samples at the root tip level (data not shown).

Endorhizal colonization by *S. algeriensis* NRRL B-24137

The strain NRRL B-24137 was easily visualized inside roots in LR white sections and in highest numbers in comparison to some parts of the root surfaces. It was visualized as hyphae inside the endorhiza of grapevine plants, 10 days post-inoculation, crossing from the rhizoplane (Fig. 3a) to inside the rhizodermis (Fig. 3a–b). The filamentous form of the strain was also visualized between the rhizodermis and the exodermis (Fig. 3b). Additionally, hyphae of the strain were visualized intercellularly between some cortical cells in the cortical zone (Fig. 3c). A pack of hyphae was also noticed intercellularly (Fig. 3d) and some cells corresponding to the strain were further visualized, intracellularly, in parts of the cortical cell layers (Fig. 3e).

Discussion

In the present study, a probe was designed for *Saccharothrix* spp. The DOPE-FISH technique has been used because of a low signal intensity, using single-labeled probe, for some microorganisms (Stoecker et al. 2010). This probe and the DOPE-FISH tool have allowed to visualize the early colonization process of *S. algeriensis* NRRL B-24137 on and inside roots of grapevine plants. Since the strain

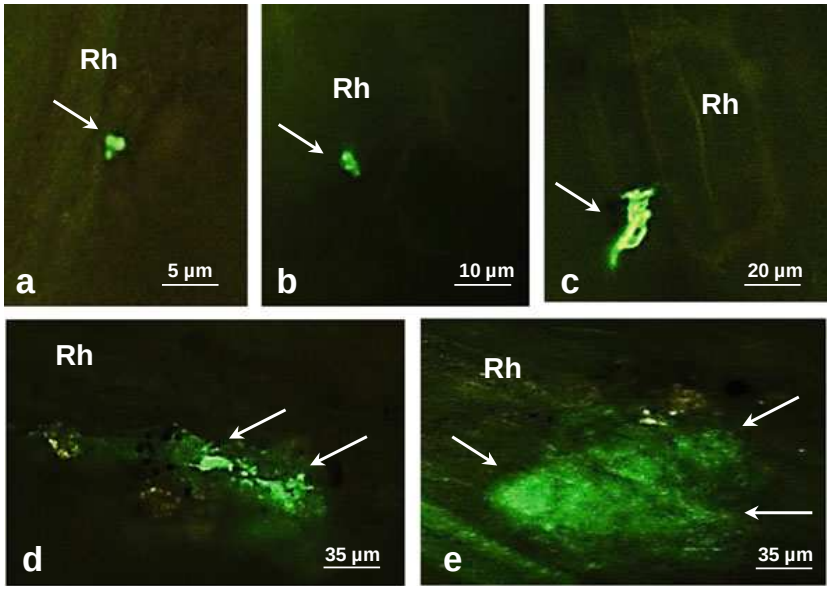

Fig. 2 Microphotographies of the grapevine root colonization by *S. algeriensis* NRRL B-24137 by DOPE-FISH microscopy at the root elongation zone showing (*arrows*) spores or germinated spores (**a**–**b**), pack of hyphae (**c**) on the rhizoplane. Parts (**d**) or the complete outline (**e**) of some rhizodermal cells were filled with bacteria. *Rh* rhizoplane

was isolated from a harsh environment, it could not be expected that it could colonize grapevine plants under climatic chamber conditions. However, it was shown in this study that the bacterium could colonize the rhizoplane of the root system of grapevine plants. The strain could colonize the root hair zone, the root elongation zone as well as root emergence site but not root tips.

Pathways of colonization have been described for different kinds of bacteria colonizing the surfaces of root systems of their hosts (Hallmann 2001; Hardoim et al. 2008; Compant et al. 2010a) as well on the grapevine rhizoplane (Compant et al. 2005, 2008). The strain NRRL B-24137 was not visualized at the root tip level, suggesting that there is no colonization of such root parts during the process of colonization. However, we cannot exclude that some bacteria, not well attached and colonizing root tips (therefore not well colonizing), were not fixed and therefore displaced. At the same time, various researches have demonstrated colonization of root tips by beneficial microbes after same kind of fixation (Chin-A-Woeng et al. 1997; Herschkovitz et al. 2005; Buddrus-Schiemann et al. 2010) and different ways of root surface colonization can be done by beneficial microorganisms (Hallmann 2001). It has been described that some bacteria will colonize preferentially the root tips, whereas for others their population pass from 0 to some populations from the tips to away and colonize rather the root hair zone. Niches providing high nutrient availability including zones of root exudation such as root hairs can be colonized by *r* strategists, whereas nutrient-poor or depleted niches will tend to be colonized by *K* strategists (Semenov et al. 1999). This

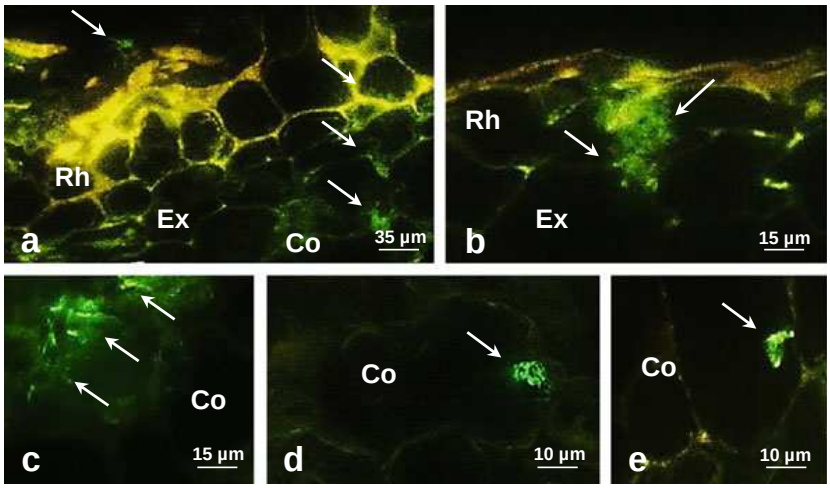

Fig. 3 Microphotographies of LR *white sections* of grapevine root colonization by *S. algeriensis* NRRL B-24137 and using by DOPE-FISH microscopy showing (*arrows*), inside the endorhiza, colonization with hyphae from the rhizodermis (**a**) to the exodermis (**a**–**b**), as well as some cortical cells colonized intercellularly (**c**–**d**) and intracellularly (**e**). *Rh* rhizoplane, *Ex* exodermis, *Co* cortical cells

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could explain why the strain NRRL B-24137 has not been detected at the root tip level, depending of its strategy to colonize grapevine plants.

The strain NRRL B-24137 was not visualized inside root hairs, but only at the surfaces of root hairs. It has been recently established that some bacteria could colonize root hair internally (Prieto et al. 2011; Mercado-Blanco and Prieto 2012), but this depends of the strain and plant–microbe interactions.

Several studies have examined the colonization process by beneficial bacteria (reviewed in Reinhold-Hurek and Hurek 1998; Compant et al. 2010a; Hardoim et al. 2008). Some can be systemic colonizers whereas others could be restricted to some root parts (Benizri et al. 2001; Hallmann 2001). Although we studied early colonization process by NRRL B-24137, we detected only colonization of this strain up to several cortical cell layers, and not in the vascular system. This suggests that the strain NRRL B-24137 will be restricted to root internal parts. However, experiments were done only 10 days post-inoculation and it may be possible then that the bacterium could reach the vascular system in longer colonizations, although this needs to be demonstrated.

The strain used in this study is an actinobacterium that could form spores and hyphae as well as pellets of hyphae (Zitouni 1995, 2004). It was, therefore, not surprising to see that during the colonization different forms of the strain could be visualized. Different studies have described colonization by actinomycetes (see for instance Coombs and Franco 2003; Merzaeva and Shirokikh 2006). In the study of Tokala et al. (2002), a *Streptomyces* sp. was visualized as spores or hyphae during the colonization of pea plants. Coombs and Franco (2003) described the colonization of a *Streptomyce*s sp. in wheat caryopses. Merzaeva and Shirokikh (2006) described colonization dynamics of *Streptomyces*, *Micromonospora* and *Streptospangium* members. Other related publications can be found regarding plant colonization by actinomycetes, especially non-Streptomycetes members (described in El-Tarabily and Sivasithamparan 2006). However, this has been never determined with the DOPE-FISH technique and a *Saccharothrix* member.

Although more work is needed to better understand the interaction between *S. algeriensis* NRRL B-24137 and grapevine plants, this study shows therefore that the strain, isolated from a Saharan soil, could colonize epi- and endophytically roots of grapevine plants as well as establish different kinds of forms during the colonization process. Although single FISH could be used to monitor the colonization process of actinobacteria, the DOPE-FISH microscopy was also used to increase the signal and to monitor the plant–microbe interaction, for which sometimes the use of other techniques restrict the visualization. This study also suggests that DOPE-FISH technique could be used to monitor plant colonization by some bacteria, such as for some specific actinomycetes.

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